REPORT DOCUMENTATION PAGE

AD-A224 478

Public reporting burden for this collection of information is estimated to average 1 hour per response, in gathering and maintaining the data needed, and completing and reviewing the collection of information. collection of information, including suggestions for reducing this burden, to Washington Headquarters S Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Pap

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE 1990	3. RE... .... Thesis/Dissertation

4. TITLE AND SUBTITLE

ANTIBODY RESPONSES IN THE NOHUMAN PRIMATE, MACACA FASCICULARIS, TO PROTEIN TOXINS

5. FUNDING NUMBERS

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

AFIT/CI/CIA - 90-052

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFIT/CI
Wright-Ptatterson AFB OH 45433

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for Public Release IAW AFR 190-1
Distribution Unlimited
ERNEST A. HAYGOOD, 1st Lt, USAF
Executive Officer, Civilian Institution Programs

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

DTIC
ELECTE
AUG 01 1990
S B D

20030206100

14. SUBJECT TERMS			15. NUMBER OF PAGES 124
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

UNCLASSIFIED

ANTIBODY RESPONSES IN THE NONHUMAN PRIMATE, MACACA FASCICULARIS, TO PROTEIN TOXINS

A
THESIS

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE

By
Geoffrey Bauman, D.M.D.

San Antonio, Texas

May 1990

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ANTIBODY RESPONSES IN THE NONHUMAN PRIMATE, MACACA FASCICULARIS, TO PROTEIN TOXINS

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May 15, 1990

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Sanford A. Miller, Ph.D.

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DEDICATION

This thesis is dedicated to the ones who so patiently tucked away their demands on my time while I worked towards the completion of this research. First, to my ever loving wife, Karen, thank you for holding up more than your share of family responsibilities. Your sacrifice bares witness to the strength of your love. As in the past, you have endured through another of your husband's "educational experiences". And to my greatest of all children in the world, Sarah and Jonathan, who did their best to understand when Daddy couldn't "come too", thank you for keeping me in your thoughts though I couldn't always be there. I'm a blessed man to have such a wonderful family.

ACKNOWLEDGEMENTS

ANTIBODY RESPONSES IN THE NONHUMAN PRIMATE, MACACA FASCICULARIS, TO PROTEIN TOXINS

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Bacterial associated protein toxins have been implicated in the pathologic processes in several diseases of man and animals. The bacterium *Actinobacillus actinomycetemcomitans* produces a protein toxin, leukotoxin, which has been defined by recent research as the primary pathologic factor associated with certain destructive periodontal diseases. Definition of the immunologic relationship between host and protein toxins may serve to identify critical factors in the scheme which might be manipulated for control of the destructive process.

The long-range goal of research in this arena is to better understand the immune response to antigenic challenge with protein toxins in man. However, for ethical reasons, some immunopathologic factors cannot be manipulated for appropriate study in man. The nonhuman primate (NhP) has, in previous investigations, been shown to be more closely immunologically related to man than any other animal model. The overall aim of this project, therefore, was to develop methodology and gather baseline data to

proceed towards a better understanding of the immune response to a protein toxin in the NhP. Tetanus toxoid (TT) was selected as the prototype protein toxin for this purpose. Response to tetanus toxoid immunization has already been widely studied in humans which allows for comparison of data obtained here to findings in man.

The specific objectives were six-fold. The first objective was to determine the validity of using antiserum developed against human IgG and IgG subclasses as a detecting agent in enzyme-linked immunosorbant assays (F'JSA) for NhP IgG and IgG subclasses. The second objective was to develop methodology for detection of anti-TT IgG and IgG subclass antibody levels as well as avidity of IgG anti-TT in the NhP serum. Third, the human directed antiserum was used to characterize the total IgG and IgG subclass response in five adult female NhPs to the protein TT. Fourth, IgG subclass proportional participation in the overall response was determined by ELISA. Fifth, a modified ELISA was developed for determination of IgG avidity in Nhp serum. Finally, avidity development of the IgG response was measured by a modified ELISA and compared to changes in total IgG antibody levels as the response matured.

When human IgG-directed antiserum was used in ELISA for detection of NhP anti-TT IgG antibody, serial serum dilution curves were very similar to that of human TT-reactive serum supporting their use as detecting agents for monkey IgG. ELISA inhibition analyses of human IgG subclass-directed antiserum for detection of monkey IgG subclasses was also justified indicating a substantial antigenic relationship of 45-85% between human and monkey IgG subclasses.

The IgG response for the five monkeys immunized with TT showed a significant increases in antibody levels over baseline with a 40-fold increase in the primary response and a 110-fold increase at peak secondary response. The major IgG subclass participants were IgG1 (62%) and IgG3 (25%) with little, though significant, responses from the IgG4 (6.5%) and IgG2 (6%) subclasses. Avidity of the IgG response also increased significantly over baseline post-primary as well as post-secondary immunization. Peak antibody levels were not coincident with peak avidity development. However, a positive correlation existed between avidity and antibody levels post immunization which was independent of time.

This investigation is the first to report on IgG subclass responses in the NhP to a protein toxin. The results afforded a preliminary look at the antibody-protein toxin interactions in an immunologically similar model to man. Tetanus toxoid is just one of many protein toxins, the leukotoxin of *A. actinomycetemcomitans* among them, responsible for pathologic processes in man. The methodology developed in this investigation and baseline information provided should facilitate future studies on the immune response in the NhP to this as well as other protein toxins.

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I. INTRODUCTION AND LITERATURE REVIEW

A. General

Animal models have been used in the past for investigation and manipulation of disease related parameters which cannot be accomplished in the human. Ideally, the mechanisms and clinical presentation of the pathologic process in question would occur similarly in the animal model and man. However, genetic variations which separate one species from another are also responsible for anatomic as well as physiologic variations. Generalization of findings from one species to another then becomes increasingly difficult as the genetic difference between animal model and man becomes greater.

The function of the immune system is in fact based on discriminating between antigenic structure of similar genetic origin and foreign matter, i.e., "self vs. non-self". The fact that the immune system can discern differences even between various members of the same species further emphasizes the complexity of animal modeling of the human immune response. Investigations involving some of the more subtle characterization of the immune response with the intent of relating the findings to man, might better be accomplished in an animal model as genetically similar to man as possible.

All vertebrates possess an humoral arm of immune defense. The humoral response in lower vertebrates often grossly simulates that of man, and can be most valuable for manipulation of large quantities of immunologic data. However, those vertebrates genetically more distant from man may vary significantly in response to various antigens. Considering that microbial pathogens in one species are often completely harmless in another; very lethal microbial toxins for man may have little affect in some animal models and vice versa. Immunologic deficiencies involving a single antibody subclass in humans can cause significant reduction in resistance to certain types of pathogens, and yet, other vertebrates may not possess the genetic code for some of the subclasses which comprise human Ig isotypes. Isotype subclasses in various animal models have substantial variation in molecular structure as well as biologic function. Subtle variations in physiologic as well as immunologic mechanisms can

result in vastly different consequences for one species versus another, and sometimes even for members of the same species.

Consideration of animals for use as models of disease involving multiple variables (i.e., immunologic, microbiologic, physiologic, etc.) should therefore take into account the level of specificity required of the animal model for generalization of findings back to the human condition.

The nonhuman primate (NhP) is the closest genetic, and therefore, immunologic relative to man (Arrington 1972). The NhP has been used previously in several disease models, and exhibited substantial similarity to man as to susceptibility to microbial agents and disease progression. Allotypic markers have been previously reported which are shared by humans and various NhPs.

As valuable as the NhP would seem as an animal model, immunologic data on the NhP is scant at this time. IgG is the major serum immunoglobulin as well as the major Ig fraction involved in many local antibody responses in the human and NhP. This fraction is also the major Ig component involved in immune memory. Investigation of the NhP IgG response to challenge with various antigenic substances would be beneficial for further definition of the immune characteristics of this model. In this investigation, methodology was developed and subsequently used to characterize the IgG and IgG subclass immune response in the cynomolgous monkey (Macaca fascicularis) to immunization with a prototype protein toxin, tetanus toxoid. In addition, avidity maturation of anti-tetanus toxoid was determined and compared to the kinetics of serum anti-tetanus toxoid levels. The immune response to this toxin has been widely studied in man, providing a wealth of data with which to compare the NhP to the host in which we are ultimately interested.

B. The NhP Model of Immune Responses

Animals have contributed highly to the well being of man in many ways, but one not often appreciated is that of providing a model in which to manipulate various parameters of disease and therapy not possible in humans. Historically, the use of animal models dates at least to the seventeenth century when blood studies were performed in the deer model by a contemporary researcher, William Harvey.

More recently, animal models have proven to be extremely valuable in immuno-
logic research (Goudsmit 1987, Anand 1984, Thanavala 1979) as well as the testing of
vaccines. Use of untested serum in man could have dire results. The laboratory
investigator using an animal model has significant advantages over colleages studying
disease in the human, since in the animal model, the moment and route of antigenic
challenge as well as extremes of dosage and cytotoxicity of measured doses can be
investigated.

The weakness inherent in modeling the human condition with animals lies in the
ability to generalize findings in the animal back to man. Failure of the system may
occur if some important aspect of the naturally occurring response is missing in the
animal model. To avoid failure of this type would requirs a knowledge of the physio-
logical, and biochemical similarities and differences of immune systems in laboratory
animal species to that of humans. Lists have been derived in which comparative
conditions, organ systems, and structures are delineated for each model to aid in ani-
mal selection (Mitruka 1976). For immunologic studies, appropriate animal selection
would involve the simplest model in which the response can be detected, consistent
histochemially and histologically with the natural occurence in man. Generally, the
less genetically related the model is to man, the greater the variation in response that
may be expected (Arrington 1972).

For this reason, the most commonly used animal models in immunology are the
mammalian vertebrates. Although significant immunologic differences are apparent
even between mammals, many investigations requiring gross simulation of the
immune response in man and manipulation of large quantities immunologic material
can often be adequately carried out with a more distant relative. Smaller mammals
are less expensive to keep, produce offspring rapidly, and are easily handled, and are
therefore often the economic model of choice. However, the subtleties of the human
immune response to particular antigens or infections, some of which are primate spe-
cific, often requires the selection of the closest immunologic relative to man, the non-
human primate (NhP). The NhP is not difficult to feed or house, but is expensive and
breeding of these animals in captivity is difficult. Nevertheless, because its close

genetic relation to man, the NhP provides a valuable animal model in the area of immunology.

An interesting outcome is revealed when we compare the availability of immunologic information in the NhP model to that of man. Whereas the animal model is normally used to develop physiologic and pathologic information for application in man, in this case much more is known about the immunologic response characteristics of man than in the NhP. The Nhp has not been used as an immunologic model to the extent of some other animals. It might seem therefore unnecessary to identify in the monkey immunologic characteristics already known in the host of ultimate interest. However, advancement of our knowledge about aspects of the human immune system may require manipulation of immunologic, microbiologic, and clinical parameters ethically not possible in man. Contrary to the usual progression of informational flow, development of the NhP model may, in this case, be guided by what is already known about the immune response in man.

The humoral response in the NhP has been investigated to a limited degree. Most of these studies have involved responses to whole microbiologic or viral entities. A competent humoral response in *Macaca arctoides* to *Actinomyces viscosus*, an organism involved in human gingivitis, for up to six months reinforced the role of the NhP as a model in the study of human gingivitis and periodontal disease (Reed 1981). The cynomolgous monkey also proved an effective model for characterization of the serum and urinary antibody responses experimental cystitis (Hopkins et al 1987). Maddison (1979) used rhesus monkeys to characterize the chronologic occurance of antibodies to *Schistosoma mansoni* infection, identifying immune protective correlative factors. Protective aspects of humoral immunity versus cell mediated immunity to measles virus infection in *Cercopithecus aethiops* monkeys was examined by Rustigian (1975). Two primate species, *Erythrocebus patas* and *Macaca mulata*, were experimentally infected with *Brugia malayi*, a human filarial parasite, and antibody levels followed by enzyme linked immunosorbant assay (ELISA). IgG and IgM antibody to the sheath of the microfilariae was associated with tha absence of the parasite in the blood, and there was and inverse relationship between antibody against filarial antigens and the

blood levels of filariae. The rhesus monkey, which is less susceptible to infection by this organism, generally had high, sustained antibody levels to the organism.

Anand et al. (1984) induced experimental immune carditis in monkeys by 12 weekly immunizations with streptococcal membrane antigen. Anti-heart antibodies increased to a maximum after the sixth injection and then declined. However, the anti-streptococcal membrane antigen antibodies appeared slightly later than the anti-heart antibodies, and continued rising until sacrifice. It was conluded that anti-streptococcal membrane antigen has a role to play in immune carditis.

Thanavala (1979) characterized the humoral response in marmoset monkeys to immunization with human chorionic gonadotropin beta-subunit and relationship of antibody levels to fertility. After primary as well as secondary immunization, animals with high antibody levels remained infertile, with increase in pregnancy positively related to decrease in antibody levels. Antibody affinity was also correlated with decreased fertility.

Pung (1988) induced Chagas'disease (*Trypansoma cruzi*) in squirrel monkeys and demonstrated similar aspects of cardiopathy as well as cellular and humoral immune response to humans. Peripheral blood monocyte showed proliferative response to epi-mastigote antigens up to twenty-four weeks post infection. Specific IgM was detected until 200 days post infection, while specific IgG remained elevated for over two years afterwards.

Lakin (1969) compared IgM and IgG precipitation, hemagglutination, and skin sensitization in *Macaca mulata* after repeated immunization with bovine serum albu-min (BSA) to previously reported findings in man. He found that the quality of passive hemagglutination and the order of magnitude of the difference seem to be similar to that found with the human anti-BSA response. Although both monkey and human IgG and IgM can act as precipitins, the IgM reaction in the monkey is a feable one. The monkey IgM anti-BSA was neither able to sensitize guinea pigs for passive cuta-neous anaphylaxis or normal monkeys for Prausnitz-Kustner type reactions.

Monte-Wicher (1970), using single radial diffusion, double diffusion gel precipitation, and quantitative precipitation test, compared antigenic similarity between

human and monkey (*Macaca mulata*) immunoglobulins IgA, IgM, and IgG. There was near identity between monkey and human IgM, somewhat less with IgG (56-60%), and still less with IgA.

The dearth of immunologic information available on such an apparently valuable animal model suggests a need for further investigation of the immune response in the monkey. IgG is the major serum immunoglobulin (Ig) in man as well as the Ig fraction most reponsible for immune memory. Although IgG and the IgG subclasses have been well characterized in other animal models, information in regard to these responses in the NhP is lacking. Other animal models can be relied on for manipula-tion of some IgG related parameters. However, as was stated previously, diversity in immunoglobulin structure as well as function appears to be related to speciation (Mota 1986).

Mouse IgG has been investigated extensively. As such, the mouse is a very valu-able model for efficient manipulation in immunologic studies. However, differences exist between the Ig of the mouse and man (Mota 1986, Golub 1987). Although the mouse has four IgG subclasses IgG1, IgG2a, IgG2b, and IgG3, they lack IgG4 and exhibit functional dissimilarities to those of man. Mouse IgG2 activates complement by both classical and alternative pathways, while IgG1 only activates the alternative pathway. Whereas mouse IgG1, IgG2, and IgG3 have three, three, and two interchain disulfide bridges, respectively, human IgG1, IgG2, and IgG3 have two, four, and four-teen, respectively. These and other problems plague comparisons between man and some of the other non-primate species.

The IgG of nonhuman primates, on the other hand, has been shown to be very antigenically similar to that of man. Terry & Fahey (1964) immunized rhesus monkeys with polyclonal IgG antisera of man. Post-immunization monkey antiserum exhibited three precipitin arcs by immunoelectrophoresis. Immunization with polyclonal human antiserum would not be expected to provide significant antigenic challenge of a single idotype to evoke an anti-idiotypic response. In addition, homologous protein sequences between human and monkey antisera would also not evoke a response. The precipitin arcs were therefore interpreted to be three groups of proteins (anti-

bodies) each exhibiting only minor differences from the monkey. Partial identity between the three precipitates and human IgG antibody was established, and three subclasses were defined suggesting protein sequence homology between monkey and human is very high.

Furthermore, NhP IgG has been proposed to contain four subclasses (Leibl 1986) similar to humans, and although NhP IgG subclasses have been studied minimally, they do appear to be similar on the basis of kappa:lambda light chain ratios. The half-life of IgG1 (@18 days), the only subclass half-life determined thus far, also appears to be similar to that of human IgG1 (Leibl 1986). Additional evidence of similarity lies in the fact that NhP IgG has been demonstrated to share some allotypic markers on the heavy chain regions with those of man (van Loghem 1986, Litwin 1969, Schuster 1969).

From the accumulated information this far, there would appear to be substantial evidence, then indicating similarity between IgG as well as the IgG subclass in the NhP and those in man. Further illumination of what appears to be a very comparable humoral system to that in man may therefore be helpful in future studies requiring the use of an immune model more representative of the human one.

C. Use of a Tetanus Toxoid for Immunogenic Challenge in the Nonhuman Primate

Tetanus toxin is produced by the organism *Clostridium tetani*, an anaerobic, spore-forming, gram-negative rod (although it may be gram-positive in young cultures). This organism was isolated in pure culture in 1889 by Kisato. Later, Behring and Kisato demonstrated that animals could be immunized with tetanus toxin which had been modified by iodine trichloride and that immunized animals' serum contains neutralizing antibodies.

The biologic function of tetanus toxin is not fully understood at this time, however several steps in pathogenesis of tetanus toxin have been determined. Tetanus toxin is released as a two-chain molecule joined by a single disulfide bond. The heavy chain is able to bind to the cell surface gangliosides and then become internalized. Whether this translocation is by some enzymatic property of TT or endocytotic action

of the cell membrane is not known although the latter is more likely. Once inside, according to current thinking, the toxin prevents presynaptic release of transmitter substance (rather than blockage of transmitter synthesis as was previously thought). The toxin (probably the native toxin) has the capability to progress retrograde in the axon towards the CNS and to actually move trans- synaptically in the process. The peripheral and central overall effect is rigidity and reflex spasms, with death most often due to respiratory failure.

Tetanus toxoid was first purified from filtrates of autolyzed cell culture in 1946 (Pillemer et al.). Originally, the molecular weight was determined to be 68 KDa with a sedimentation constant of 4.5S, however more contemporary studies have recently indicated a sedimentation constant of about 160 kDa (Matsuda 1975). Tetanus toxin is a heat labile polypeptide which can be trypsinized into 2 fragments of approximately 107 kDa and 53 kDa. The larger fragment appears to be associated with the toxic properties, however toxicity is significantly diminished by fragmentation. The smaller, nontoxic fragment has been subdivided by exposure to a reducing agent into two segments of approximately 23 and 32 kDa suggesting the fragments were joined by a disulfide bond (Robinson 1978).

Genetic information for tetanus toxin synthesis is plasmid associated. The tetanus toxoid gene coding sequence has been cloned for overlapping portions of the molecule (Fairweather 1986, Eisel 1986) which thereby provided a comple*e amino acid sequence for the molecule. The protein contains 1,315 amino acids and has a molecular weight of 150,700. After splitting light and heavy chains, the molecular weight of each is 52,288 and 98,300. Survey by computer comparison of amino acid sequences of tetanus toxoid to that of thousands of other proteins show no significant homology with other proteins. Furthermore, there is no duplication of amino acid sequences throughout the entire molecule.

Studies by Robinson (1974, 1982) indicate a highly ordered secondary structure. Alpha-helical content of the native chain, versus the light chain, versus heavy chain was 35%, 24% and 30% respectively. However, the Beta-structure content was 30%, 2%, and <1%. This would indicate that helical structure is 80% maintained when the

native molecule is split versus very minimal beta structure. Computer derived prediction secondary structure shows a beta rich region at C- as well as N-termini, and a central region rich in alpha helix, especially towards the C-terminus (Chou & Fasman 1978). Hydrophobicity analysis has not demonstrated a large locus of hydrophobicity as would have been expected by the toxin's ability to interact with nerve cell membrane with internalization of the molecule. Smaller loci of hyrophobicity are apparent randomly distributed over the surface of the molecule, however only two long enough to span animal cell membranes. One is in the light chain and the other in the heavy chain. Tertiary structure is suggested to be very stable by rotational strengths of major bands by near-UV circular dichroic spectra evaluation.

Studies with polyclonal antiserum to tetanus toxoid demonstrated that although various lesser mammals (horse, rabbit, mouse, guinea pig) identify epitopes on the light chain, human anti-TT identifies only epitopes on the heavy chain (Matsuda 1983). This may support a role for the heavy chain in toxic activity.

Tetanus toxoid was selected for immunization of the NhP model because it has been widely studied in man providing a wealth of data with which to compare results in the relatively uncharacterized NhP (Sepp 1984, Kim 1989, Baraff 1986). Immunologic manipulations in the NhP, not possible in man, may then further describe relationships in man to tetanus toxoid as well as to other protein toxins which may be antigenically related. Because tetanus toxoid is so widely used therapeutically in the patient population, the immune responses in humans with accompanying conditions has been broadly reported in the literature. Tetanus toxoid has been used to describe variations in the human immune response in various human disease states such as tuberculosis (Al-Tawil 1978), asthmatic patients (Grove 1975), Hodgkin's disease (Fisher 1985), Crohn's disease (Stevens 1985), and others. It has also been used to assess immune variation during various therapies and normal physiologic changes such as during pregnancy (Rao 1980), neonatal status (Einhorn 1987), age related decline in antibody synthesis (Kishimoto 1980), steroid therapy (Johnson 1987), post bone marrow transplantation (Shiobara 1986), and others. This unique position of knowing more about the immunologic response in the human than in the model may

allow for extrapolation of information back to the model to guide manipulation of immunologic material, not possible in the man, furthering the understanding the human response.

D. Humoral Immune Response in Periodontal Disease

Most of the substances elaborated by dental plaque are antigenic and therefore evoke an immune response. The response is made up of cellular and humoral components. For the subject matter of this thesis, the humoral aspects deserve further discussion. Immunoglobulins, or antibodies, comprise the humoral arm of the immunity. There are 5 classes or isotypes in humans (IgM, IgG, IgA, IgD, and IgE) of which IgG makes up the largest proportion. Antibodies participate in the immune response by one of the following mechanisms: 1) activation of the *complement system* (IgG1, IgG3, IgM) leading to lysis or augmenting PMN/macrophage efficiency through opsonization of antigens; 2) marking of foreign cells for recognition by natural killer lymphocytes; 3) release of inflammatory mediators (IgE); and prevention of the antigen adherence (i.e. microorganism) to its target (Male 1986).

Studies by Mackler and co-workers suggest that the immune response in gingivitis is predominantly mediated by T-lymphocytes whereas that of periodontitis is B-cells (Mackler 1977, 1978a, 1978b). While biopsies from gingivitis sites contained 94% T- cells, biopsies from periodontitis sites revealed a predominance of lymphocytes (78% IgG, 9% IgM, and 4% IgA) and plasma cells (67% IgG, 24% IgM, and 8% IgA). Severe gingivitis was considered to be a transitional phase between these 2 entities with about 50% B- lymphocytes primarily from the IgG1 and IgG3 subclasses. Characterization of the regional aspects of the inflamed connective tissue adjacent to the periodontal lesion was carried out by Johannessen (1986) with monoclonal antibodies. Deeper areas (periodontitis) of the lesion were dominated by B-lymphocytes whereas the shallower areas (gingivitis) contained more T-lymphocytes. These findings are consistent with the hypothesis that periodontitis is a "B-cell lesion".

Until recently it was believed that crevicular fluid antibody was merely a dilution of serum immunoglobulin in which the volume of fluid reflected the degree of local inflammation (Scheinkein 1977a). If this were true, then sites with similar levels of

inflammation throughout the mouth should demonstrate similar levels of crevicular fluid antibody specific for local antigenic stimuli (microbial) at some level lower than that of serum. Ebersole et al. (1985) as well as others (Lally 1980, Lovelace 1982) determined, however, that individual sites throughout the mouth are not only highly variable in specific antibody level, but are often actually higher than that of serum which is consistent with local production of specific immunoglobulin.

Ogawa (1989) demonstrated resident plasma cells actively secreting immunoglobulins within chronically inflamed gingival tissue. By enzyme-linked immunospot, IgG was the predominant isotype produced in periodontal disease tissues, followed by IgA. An increase in plasma cell populations was noted with an increase in disease severity. Careful analysis showed that although absolute levels were greater, the distribution of the IgG and IgA isotype plasma cell populations in the gingiva were similar to those found in the synovia of rheumatoid arthritis patients, normal lymphoid tissues, and serum.

Another study by Ebersole and co-workers (1985) demonstrated the dynamic response relationship between local and systemic antibody response. Levels of crevicular fluid antibody and homologous serum antibody were recorded over time in 61 patients with various forms of periodontal disease. Fluctuation in levels of local periodontopathogens versus local antibody, versus systemic levels were compared as patients passed from active to inactive and back to active disease status. The most likely hypothetical sequence of events suggested by events in this study was: 1) infection, 2) local antibody response, 3) disease activity, 4) systemic antibody response which remains at elevated levels after the disease is brought under control. Local antibody response then would assume a major role in controlling the initial infection. Infection at other sites might later be controlled by systemic immune anamnestic responses. The importance of local antibody production against crevicular pathogens is thus evident.

E. *Immunoglobulin G and IgG Subclasses*

Besides being the major component of serum immunoglobulin (13.5 mg/ml in man) (Golub 1987), IgG is the primary immunoglobulin involved in long term immu-

nity and immune memory (Male 1986). It is also the predominant immunoglobulin of gingival crevicular fluid (Ogawa 1989, Okada 1983, Mackler 1977). With a molecular weight of about 150,000, one molecule of IgG is made up of 2 light chains (~25,000 mw each) and 2 heavy chains (~50,000 mw each). The molecule is grossly "Y" shaped with the antigen binding function residing in the forked end of the "Y" (Fab portion) while the single tailed end of the Y (Fc portion) functions in complement activation, phagocytic cell attachment (opsonization), and clearance. If the length of the "Y" shaped molecule were divided four segments, the quarter of the Y comprising the tips of the 2-tailed end contains the most variable portion of molecule and is called the variable region. The remainder of the molecule is known as the constant region. Only heavy chains are found in the Fc portion of the molecule, while the Fab portion is made up of two light chains and the extensions of the heavy chains in the Fc portion. This factor is important in isotype and subclass identification.

Although major differences within and between antibodies resides in the variable regions, the loci of minor differences in the constant regions specifically identify each of the isotypes (70% heterology) or subclasses (5% heterology) from the others. These relatively minor variations are the epitopes against which monoclonal antisera are directed for identification of a particular isotype or subclass (Terry 1964). The heavy chains, therefore, are classified as α (IgA), δ (IgD), ε (IgE), μ (IgM), and γ (IgG) for the five isotypes, and γ1, γ2, γ3, and γ4 for the four subclasses of IgG.

The discovery by Grey & Kunkle (1964) and Terry & Fahey (1964) of the IgG subclasses was a relatively recent achievement. As mentioned previously, the dissimilarity between the subclasses is related to amino acid variations of the heavy chains. These variations are located, for the most part, in the hinge region; however, and therefore can result in conformational differences. As structure tends to govern function, there are significant functional variations. Only IgG1 and IgG3 (with some minor participation of IgG2) can activate complement. Although all subclasses appear to be synthesized at a similar rate (33 mg/kg/day), the half-life of IgG3 is one third that of the other subclasses (7 days versus 21 days respectively). This is effected by a greater rate of catabolism of IgG3 (Golub 1987). One of the most significant functional differ-

ences between the subclasses is restriction of responses related to antigenic chemical make-up (Hammarstrom 1986). In normal adults, there appears to be two distinct patterns of IgG subclass response. Protein antigens will induce mainly an IgG1 response with minor contributions from IgG3 and IgG4, whereas polysaccharide (including lipopolysaccharide) antigens evoke a response primarily of the IgG2 subclass. In IgG2 deficiency states, frequent respiratory infections often result from bacteria possessing protective polysaccharide capsules (Hammarstrom 1986). Altered patterns of subclass responses to various antigens may also be noted in children. Since the IgG2 and IgG4 subclasses are not developed until adolescence, the subclass response to polysaccharides is derived from the IgG1 subclass (Oxelius 1979) although these antibodies may be of a lower affinity for the polysaccharide antigen than would be IgG2. IgG4 antibodies appear to be increased in conditions of chronic antigenic stimulation (Aalberse 1983). Desensitization of patients to bee venom or dust mite involves chronic exposure to the particular antigen. Patients in which desensitization is effective develop a significantly increased IgG4 response which seems to protect against IgE mediated mast cell degranulation. A significantly increased IgG4 response may therefore be detected in an individual to a protein antigen after repeated natural or clinical exposure to that protein. In addition, a host of immunodeficiency disorders as well as systemic pathologies can potentially alter IgG response levels (Karger 1986) but are beyond the scope of this discussion.

Ogawa (1989) assessed subclass participation in the local antibody response during periodontitis. IgG was the predominant isotype, followed by IgA. The major subclass participant was IgG1 followed by IgG2 with lesser, but similar levels of IgG3 and IgG4. IgA1 predominated over IgA2 in moderate stages of disease; however, IgG4 and IgA2 levels increased in the advanced stages of periodontitis.

F. IgG Subclasses in Animal Models

1. Selection of the Animal Model

Immunoglobulins have been identified in all vertebrates investigated and diversity in structure appears to be related to speciation (Mota 1986). In lieu of this, more closely related animals seem to exhibit more homology in Ig structure.

Mouse IgG has been investigated extensively. As such, the mouse is a very valuable model for efficient manipulation in immunologic studies. However, differences exist between the Ig of the mouse and man (Mota 1986, Golub 1987). Although the mouse has four IgG subclasses IgG1, IgG2a, IgG2b, and IgG3, they lack IgG4 and exhibit functional dissimilarities to those of man. Mouse IgG2 activates complement by both classical and alternative pathways, while IgG1 only activates the alternative pathway. Whereas mouse IgG1, IgG2, and IgG3 have three, three, and two interchain disulfide bridges, respectively, human IgG1, IgG2, and IgG3 have two, four, and fourteen, respectively. These and other problems plague comparisons between man and other non-primate species.

Terry & Fahey (1964) immunized rhesus monkeys with polyclonal IgG from human serum. Post-immunization monkey antiserum exhibited three precipitin arcs by immunoelectrophoresis. Immunization with polyclonal human antiserum would not be expected to provide significant antigenic challenge with a single isotype to evoke an anti-idiotypic response. In addition, homologous protein sequences between human and monkey antisera would also not evoke an immune response. The precipitin arcs were therefore interpreted to be three groups of proteins (antibodies) each exhibiting only minor differences between the humans and monkeys. Partial identity between the three precipitates and human IgG antibody was established, and three subclasses were defined suggesting protein sequence homology between monkey and human existed. However, presently there is minimal information in the literature which has effectively identified the presence of IgG suclasses in *M. fascicularis*. Thus, currently the existence and functional capabilities of these molecules remain to be elucidated.

2. Use of Mouse Anti-human IgG Monoclonals to Detect IgG Subclasses in the NhP

In accordance with studies reporting considerable structural similarities between monkey and human IgG subclasses, mouse anti-human subclass IgG monoclonal antibodies have been used in previous investigations to characterize the IgG subclass response in the nonhuman primate. Although cross reactivity of

human IgG directed monoclonals has, in fact, been reported with IgG epitopes of other mammalian species (Jefferis 1982), there was no cross-reactivity of subclass specific antibody to IgG1, IgG2, and IgG3 (Reimer 1984). Cross reactivity of a few types of monoclonals between species has been noted for IgG4 however. Data which would help to clarify the ability of anti-human monoclonals to detect immunoglobulin molecules in the serum of cynomolgous monkeys would be critical for characterizing the dynamics of the antibody response to immunization.

The enzyme-linked immunosorbant assay (ELISA) has proven to be among the most sensitive assays for quantitation of antibody levels (Djurup & Weeke 1986), and is very familiar to our laboratory. The use of polyclonal antisera has been shown to be very adequate for detection of antibody isotype levels. However, for greater specificity, the use of monoclonal antibodies is much to be preferred for detection of individual antibody subclasses for solid phase techniques (Djurup & Weeke 1986). For this reason, nonhuman primate and human antibody responses were compared during this investigation using an ELISA to ascertain the similarity of IgG molecules as determined by antibody binding curves and antibody-antigen inhibition curves.

G. Antibody Avidity (Affinity)

Recently, it has become evident that increased antibody titers, although important, are often not sufficient to prevent or curtail disease. Effective antibody response requires adequate binding (avidity) of the antigen by the antibody. The importance of antibody avidity has been demonstrated by several studies (Steward & Steensgard 1983, Brown 1984, Noble 1987). Brown (1984), discovered that the affinity of antibodies to hepatitis B surface antigen were significantly higher in patients who recovered from the disease than in those who continued with chronic hepatitis. Devey (1985, 1988) determined that the IgG subclass response to immunization with tetanus toxoid showed significant differences in antibody avidity between IgG1 and IgG4 subclasses. Most patients demonstrated a dominance of IgG1 subclass in the response. However, some individuals also showed a very high level of IgG4 subclass antibodies. Avidity of the overall IgG response in these IgG4 high responders was lower than that of patients

with predominantly IgG1. Further investigation determined that significant decreases in IgG4 avidity was the determining factor in the finding.

Antibodies are sometimes compared to enzymes because each has a specific site into which a particular chemical must fit in order for activity to occur. An additional similarity is that although the enzyme is ultimately specific (only one specific chemical fits best the active site), the antibody may also have several near fits. In fact the antibody population, in general, is very non-homogeneous in binding site specificity and strength. This property is responsible for a wide variety of association constants which describe antibody-antigen interactions. Rather than describing a particular association constant to describe avidity, the "average" association constants, are considered which comprise the array of interactions between the assorted antibody binding sites and target sites (epitopes) on the antigen. Eisen (1964) investigated the development of antibody avidity in rabbits after immunization with dinitrophenyl bovine gamma-globulin (DNP-BGG) using equilibrium dialysis and fluorescence quenching methodology. Results indicated that average association constants were distributed over a broad range of activities. In addition, development of avidity was dependent on antigenic dose at immunization and with time post immunization. When large amounts of antigen were used for immunization, average association constants were low and remained so with time post immunization. When small immunizing doses were given however, antibodies of high affinity and great heterogeneity in association constants prevailed, and affinity increased significantly with time post immunization. Intermediate dose concentrations resulted in avidities and heterogeneity of association constants between these two extremes. Eisen offered the following explanation for the increase of avidity with time post immunization: 1) Initial challenge with low levels of antibody would preferentially result in interaction with high affinity antibody receptors on B cells. The complexes formed would initially be cleared rapidly leaving relatively little antigen stimulation of lower affinity antibodies; and the high affinity antibody associated responses, however, having been stimulated would result in high levels of antibodies with greater affinity as the response developed.

Several methods have been used in the past to investigate antibody avidity development post immunization. However, most of these technique are cumbersome and equipment intensive. Pullen (1986) introduced a variation of the standard ELISA to determine antibody avidity. A chaotropic ion, in this case ammonium thiocyanate, over a range of molarities, interrupts antibody-antigen binding. An avidity index was computed based on the molar concentration of eluant which was necessary to result in a 50% reduction in antigen-antibody interaction. MacDonald (1988) compared this modified ELISA to a very accurate contemporary but more cumbersome technique (equilibrium dialysis) and demonstrated similar results with both methods. Thus, this technique was developed for studies examining the maturation of the systemic antibody response in NhP following active immunization with a protein toxin.

H. Statement of the Problem

Bacteria have long been implicated as participants in the destructive process of periodontal disease. Although recent studies have unequivocally shown bacterial plaque to be the primary etiologic agent in periodontitis (Loe 1965, Loesch 1976), a myriad of microbial and host factors participate in the environment of the periodontal lesion. Periodontitis has been defined as a multi-factorial disease, and identification of the most important factors from this complex environment has proven to be a formidable task.

The nonhuman primate appears to respond immunologically in many ways similar to man. Since manipulation of periodontitis associated pathogenic and host factors in man is ethically and legally impractical, the monkey model offers advantages in characterizing the response to this protein toxin. Although other animal models have been used successfully to study periodontopathogenic processes, the protein LT is only active against leukocytes of man and some nonhuman primates.

The rationale for this investigation is based on the following principals: 1) sufficient evidence exists that protein toxins LT are important agents in the destructive processes of periodontitis; 2) substances which are structurally similar (i.e., proteins, polysaccharides, etc.) appear to elicit grossly similar humoral responses relative to timing of response and subclass restriction, 3) the nonhuman primate demonstrates

similar immune characteristics to man; and 4) characterization of the immune response in the nonhuman primate to tetanus toxoid, a widely available toxin which has been used in several human studies, should shed some light on the characteristics of the response in the NhP and man to protein toxins such as LT.

This investigation addresses: 1) development of methodology for characterization of IgG and IgG subclass antibody level and avidity in nonhuman primate serum, and 2) characterization of IgG and IgG subclass development and avidity following active immunization with a prototype toxin in the nonhuman primate. Some major participants in the pathologic process have been determined recently. In particular, the identification of A. actinomycetemcomitans as the dominant organism in a particular periodontal disease (localized juvenile periodontitis), and the fact that the most pathogenic strains of A. actinomycetemcomitans are those which elaborate LT provide evidence of LT involvement in this disease. Neutralization of leukotoxin activity by serum anti-LT antibodies further implicates LT in the destructive process. Information gained from this study may be used to guide future investigation for characterization of the nonhuman primate immune responses to protein toxins and development of immunological strategies to interfere with their function.

II. MATERIALS AND METHODS

A. Animals

Seven adult year old female cynomolgus monkeys (M79, M99, N21, N45, P61, P69, and R25) were obtained from Charles River Laboratories (Port Washington, N.J.). All monkeys were quarantined for 12 weeks upon arrival before any experimental procedures were initiated. Procedures involving sedation of the monkeys were accomplished by intramuscular ketamine injection (7 mg/Kg). Monkeys were individually housed and fed a soft chow (Primate Diet^R) and water ad libitum.

B. Tetanus Toxoid (Protein Toxin)

Tetanus toxoid fluid (Ultrafined^R, Wyeth Labs), which was 97% free of non-toxoid nitrogen, was used for all immunizations. The concentration of tetanus toxoid in this preparation was 200 µg/ml. This tetanus toxoid is prepared by growing a suitable strain of Clostridium tetani on a protein-free semisynthetic medium . Formaldehyde is used as the detoxifying agent for the tetanus toxin. The final product contains no more than 0.02% free formaldehyde and contains 0.01% thimerosal as preservative. Protein concentration of this preparation was determined using a BCA protein assay system (Pierce Biochemical Co.)

A more concentrated tetanus toxoid preparation was used as the antigen source for development of ELISA antibody detection procedures. This preparation was graciously provided by Wyeth Laboratories for this investigation. Protein concentration of this preparation was also analyzed by BCA and determined to be 3.98 mg/ml.

C. Immunization Protocol

After collecting baseline serum samples from all monkeys, two (N21 and N45) were selected for providing immune sera. Each reference monkey was immunized subcutaneously in the proximal, dorsal area of the hindlimb with 0.5 ml Tetanus Toxoid UltrafinedR. Repeat immunization of reference monkeys was accomplished two weeks later in the opposite hindlimb. The experimental protocol included monkeys

(M79, M99, P61, P69, and R25) which were immunized after baseline sera collection and again one month later with the same dose and route of antigen as the reference animals.

D. Serum Collection and Preparation

Serum from the reference monkeys (N21 and N45) was obtained at two weeks post immunization and one and two weeks after the second immunization (four weeks post- baseline). Serum from the experimental monkeys was collected at baseline, 7, and 30 days post primary immunization, and 3, 7, 30, 60, 90, and 120 days post secondary immunization.

Three to five ml/monkey of whole blood was collected from sedated animals using venipuncture of the femoral vein. Blood was allowed to clot at room temperature and then placed at 4°C overnight for clot retraction. Cells were removed by centrifugation (3000 x g; 15 min) and serum was stored at -20°C in 2 ml aliquots until used for the various investigative procedures. Reference monkey sera from post primary and secondary immunizations were combined to provide a single reference standard for all ELISA analysis.

E. Detection of NhP IgG & IgG Subclasses with Human Directed Antiserum

The various types of experiments were performed to delineate the relationship between IgG and IgG subclass molecules in human and nonhuman primate sera, as well as to define the specificity of the murine monoclonal antibodies that were utilized throughout these studies. Each of these experiments were designed to examine the ability of the MoAb to bind to purified human myeloma proteins of each subclass. Also, the ability of molecules with similar or identical antigen epitopes in human or NhP sera to competitively inhibit the reaction with the monoclonals was tested.

The first study was designed to examine the ability of affinity purified goat anti-human IgG (GAHG; Calbiochem) to detect antibodies to TT in both human and non-human primate sera. For this study, plates were coated with TT as antigen (section F.1) and incubated with dilutions of a human serum containing high levels of antibody to TT (1:200-1:102,400) or with serum from NhP actively immunized with TT (1:200-1:102,400). The plates were developed with GAHG at a 1:500 dilution and sub-

sequent reagents as described in F.1.

The initial experiments for assessing subclass relationships utilized ELISA plates coated with amounts of human myeloma IgG1, IgG2, IgG3 or IgG4 (Calbiochem) from 10 µg to 100 pg. These were dissolved in buffer I and incubated in the wells as described in the succeeding section. Unbound myeloma protein was removed by washing (buffer II) and incubated with a mouse monoclonal antibody to human IgG1 (Calbiochem HP6001), IgG2 (Calbiochem HP6002), IgG3 (Calbiochem HP6047), or IgG4 (Calbiochem HP6025) at a dilution of 1:1000, 1:400, 1:1000, and 1:800, respectively. The antibodies were diluted in 0.5% nonfat dry milk (Sanalac^R) in buffer III (100 µl/well) to minimize background. After a two hour incubation on a rotator at room temperature, the plates were washed and biotinylated goat anti-mouse IgG (GAMG) (1:400) (Zymed) in 0.5% blotto-BIII was added to each plate (100 µl/well). Plates were again incubated for two hours on a rotator at room temperature and washed and tapped dry to remove excess GAMG. Streptavidin conjugated with alkaline phosphatase (SA-AP) (Zymed) in 0.5% blotto-BIII (1:1000) was added (100 µl/well) and the plates were incubated on a rotator at room temperature overnight. The plates were again washed to remove unbound SA-AP, and the colorless enzyme substrate, p-nitrophenylphosphate (NPP) at a concentration of 1 mg/ml in buffer IV was added as a substrate (200 µl/well). The reaction was stopped with 1N NaOH (100 µl/well) at 30 minutes and the extent of reaction was determined spectrophotometrically at 410 nm (Dynatech MR650). Additional wells were coincubated with the mouse monoclonal antibodies and either human serum or nonhuman primate serum. The human serum was previously characterized for the total level of each IgG subclass as compared to the WHO 67/97 reference standard. This serum contained 8.63 mg/ml IgG1, 3.96 mg/ml IgG2, 0.427 mg/ml IgG3, and 0.304 of IgG4. The human serum added in this competitive inhibition experiment was diluted so as to contain from 6-8 µg of each subclass. In the absence of information on absolute amounts of each subclass in the NhP sera, this was utilized in the inhibition assays at a dilution identical to the human serum for each subclass. All other incubations and developing steps were identical. All analyses were performed in triplicate.

The second set of experiments utilized serum from NhP that had been actively immunized with TT, as well as a human serum that had previously been characterized to contain high levels of anti-TT IgG antibody activity. ELISA plates were coated with TT as antigen (section F.1). Triplicate wells were incubated with varying dilutions of either the human or NhP serum (1:50-1:40,000) and incubated to allow antibody binding. The amount of IgG subclass antibody activity was then determined by development of the system with specific monoclonal antibody. After allowing binding of antibody from the serum, additional wells were co-incubated with the subclass specific MoAb and 100 ng/well of the homologous subclass myeloma protein. This competitive inhibition was designed to examine the ability of a human IgG subclass protein to block the reaction between the anti-human MoAb and antigenically similar molecules in the human and NhP sera that expressed antibody reactivity to TT. Similar dynamics of inhibition would support that the molecules detected in this system expressed some antigenic relationship.

The final set of experiments again utilized TT coated ELISA plates and the human and NhP sera with antibody activity to TT. In this study a dilution of the human (1:1000-IgG1; 1:200-IgG2; 1:500-IgG3; 1:200-IgG4) or NhP (1:100-IgG1; 1:50-IgG2; 1:400-IgG3; 1:50-IgG4) antibody containing serum was incubated in the plates to allow binding the IgG anti-TT antibody. The level of antibody activity was then determined by development with the appropriate MoAb and subsequent enzyme containing reagents. Additional wells were analyzed by co-incubation of the MoAb with a human serum (very low level of anti-TT IgG antibody) at a dilution containing 1 µg of the IgG subclass. Similar wells were analyzed using a dilution of an NhP serum (no anti-TT IgG antibody) that corresponded to the dilution of the human serum. The system was developed in a similar fashion and the level of inhibition compared to the control wells in which no competitive serum was added.

F. Anti-TT Antibody Analyses (Enzyme-Linked Immunosorbent Assays)

The Enzyme-Linked Immunosorbant Assay (ELISA) is an immunologic assay which can quantitate specific antibody isotypes and even isotype subclasses in biologic fluids. An antigen is bound to a solid phase, after which the solid phase is exposed to

the antibody' containing fluid under investigation. Antibodies specific for the particular solid phase attached antigen will complex with the antigen. The solid phase is then rinsed, removing all except the antigen-antibody' complexes. Next, an enzyme conjugated antisera is added to the solid phase environment. This antisera is specific for the particular antibody' isotype (or isotype subclass) participating in the solid phase attached antigen-antibody' complexes. The result is a solid phase-attached antigen-antibody'-antibody'-enzyme complex which remains when the solid phase is again rinsed. The enzyme is provided with a substrate whose conversion results in a colorimetric change dependent on the quantity of enzyme and proportionately to the experimental serum antibody present.

The amount of change is then determined spectrophotometrically and reported as a change in optical density. Engvall and Perlmann (1972) were the originators of the ELISA technique which was adapted to microtiter plates by Voller et al. (1974) ELISA procedures for this investigation were based on modifications of the methodology of Ebersole et al. (1980). The antibody levels are expressed as ELISA units (EU) which are determined in relation to a linear regression plot of serial dilutions of a reference standard serum. The standard (or reference) serum is assigned a relative value of 100 ELISA units at a set of dilutions which provides a broad OD range. Reference serum linear regression curves were defined by relating the OD410nm to $\log_{10}$ EU.

1. Total IgG Antibody Response

ELISA development for total IgG is described schematically in **Figure 1.** Immulon polystyrene 96 well microtiter plates (Dynatech) were coated with tetanus toxoid (0.75 µg/well) at a concentration of 5 µg/ml in buffer I (see appendix A). The plates were incubated at 37°C for 3-4 hours and then stored at 4°C at least overnight or until used. Prior to addition of serum samples, the plates were washed five times with PBS to remove unbound tetanus toxoid. The plates were tapped dry and serum samples added in triplicate at concentrations of 1:100 and 1:200 (100µl/well) in Buffer III. Each plate contained representative serum samples from all bleedings for a single monkey to provide a more accurate characterization of the immune response in that animal. Reference serum was applied in duplicate

FIGURE 1.

Protocol for detection of total IgG anti-tetanus toxoid antibody levels in NhP serum.

ELISA for Detection of NhP IgG Serum Antibody

Tetanus Toxoid

$37^{\circ}C, 4^{\circ}C$

▼

NhP serum diluted in PBS/Tween 20

2 h
wash

▼

Goat anti-human IgG

2 h
wash

▼

Rab anti-Goat IgG/AP

16-18 h
wash

▼

NPP Substrate

30 min.

▼

NaOH

▼

OD 410 nm

starting with a 1:100 dilution and serially diluting two fold through 1:800. Next the plates were incubated at room temperature on a rotator for 2 hours. Plates were then washed with PBS and tapped dry as previously described to remove unbound sera, after which goat anti-human IgG (GAHG) (Calbiochem) was added to all wells (100 µl/well) at a dilution of 1:500. The plates were again incubated on a rotator for two hours at room temperature. Plates were washed as before to remove unbound GAHG, and 100 µl/well of rabbit anti-goat IgG conjugated with alkaline phosphatase (RAGG-AP) (Sigma Chemical) was added (100 µl/well) at a concentration of 1:800. The plates were then incubated overnight on a rotator at room temperature. The following day, the plates were washed as before to remove unbound RAGG-AP, and the colorless enzyme substrate, p-nitrophenylphosphate (NNP) (Sigma 104R), was diluted in Buffer IV (see appendix A) at a concentration of 1 mg/ml and added as substrate (200 µl/well). Reduction of the NNP to p-nitrophenol by bound alkaline phosphatase produces a yellow color which increases in intensity until the reaction is stopped by the addition of 1N NaOH (100 µl/well). The reaction was stopped in this manner at 30 minutes and the extent of reaction was determined spectrophotometrically at 410 nm (Dynatech MR650).

2. IgG Subclass Antibody Response

IgG subclass quantitation was accomplished on Immulon 96 well microtiter plates which were antigen coated with tetanus toxoid (15 µg/well) diluted in Buffer I (100 µg/ml)(**Figure 2**) These plates were incubated at 37°C for 3.5 hours and then stored at 4°C at least overnight or until used.

Plates were washed 5 times immediately prior to usage with PBS to remove unbound antigen, and then tapped dry. 0.1% gelatin blocking agent in Buffer III was added to all wells (150 µl/well), and plates were incubated on a rotator at room temperature for two hours. Serum samples were applied to the plate in triplicate at concentrations of 1:10 and 1:25 in Buffer III. Each plate contained representative serum samples from all time points for a single monkey. Reference serum was applied in duplicate to all plates in two fold dilutions at concentrations of 1:10 though 1:80 for inter-plate comparison. The plates were then incubated for

2 7

FIGURE 2.

Protocol for detection of IgG subclass anti-tetanus toxoid antibody levels in NhP serum.

ELISA for Detection of NhP IgG Subclass Serum Antibody

Tetanus Toxoid

▼ $37^\circ C, 4^\circ C$

**NhP serum diluted
in PBS/Tween 20**

▼ *2 h
wash*

**MoAb anti-human IgG1,2,3,4
in 0.5% nonfat milk**

▼ *2 h
wash*

**Goat anti-Mouse IgG/Biotin
in 0.5% nonfat milk**

▼ *2 h
wash*

**Streptavidin/AP
in 0.5% nonfat milk**

▼ *16-18 h
wash*

NPP Substrate

▼ *30 min.*

NaOH

▼

OD 410 nm

two hours on a rotator at room temperature. Plates were washed as before to remove unbound sera, tapped dry, and received the appropriate concentration of mouse anti-human IgG1 (Calbiochem HP6001), IgG2 (Calbiochem HP6002), IgG3 (Calbiochem HP6047), or IgG4 (Calbiochem HP6025) monoclonal antibodies (MAHGs) (1:1000, 1:400, 1:1000, 1:800, respectively) in 0.5% nonfat dry milk (Sanalac^R) in Buffer III (100 µl/well). Following a two hour incubation on a rotator at room temperature, plates were washed and dried as previously described, and biotinylated goat anti-mouse IgG (GAMG) (1:400) (Zymed) in 0.5% blotto-Buffer III was added to each plate (100 µl/well). Plates were again incubated for two hours on a rotator at room temperature and washed and tapped dry as before to remove excess GAMG. 100 µl/well Streptavidin conjugated with alkaline phosphatase (SA-AP) (Zymed) in 0.5% blotto-Buffer III (1:1000) was added and the plates were incubated on a rotator at room temperature overnight.

The following day, the plates were again washed to remove unbound SA-AP, and the colorless enzyme substrate, p-nitrophenylphosphate (NPP) in Buffer IV (1 mg/ml) at a concentration of 1 mg/ml was added as a substrate (200 µl/well). The reaction was stopped with 1N NaOH (100 µl/well) at 30 minutes and the extent of reaction was determined spectrophotometrically at 410 nm (Dynatech MR650).

Antibody levels are reported in ELISA Units (EU's) as relative concentrations which were determined by comparison to a reference serum linear regression curve which was defined by relating the OD to $\log_{10}$ EU.

3. Subclass Proportional Response

This ELISA was performed similarly to that for subclass level determination (**Figure 2**) except as will be described. After TT coating of a microtiter plate, reference sera was added at equal concentrations in all wells (except background wells). Mouse monoclonal antibodies (MAHG 1,2,3, & 4) were then added in duplicate at a very high concentration in the first wells (column 1 on the plate) and diluted serially 2 fold. At high monoclonal concentrations applied in initial columns, an excess of the monoclonal antibody was achieved. Since all wells contained equal concentrations of nonhuman primate serum, there was a limited

amount of antibody present with which the monoclonals could react. In those wells where maximum monoclonal-antiserum interaction had occurred, excess monoclonals were washed from the plate during the procedure. Since the reactions between NhP antibody - MAHG - GAMG - SA-AP occur in a proportional ratio, the OD at the point of maximum antiserum-monoclonal reactivity can be interpreted as representative of the proportion of each subclass in the reference serum. Knowledge of the inter-subclass relationship of the reference serum (which was included on all ELISA plates) therefore allows extrapolation to provide comparison of levels between and within plates containing the different subclasses.

Maximum OD's (OD at maximum antiserum-monoclonal activity) for the various subclasses in the reference sera were related such that the lowest maximum OD was arbitrarily assigned a factor of 1 and the others were then multiples of this as related to the inter-OD relationship. IgG2 was the lowest responder and was assigned a factor of 1; IgG4 had a relative factor of 1.2; IgG3 was at a relative level of 3.0, and IgG1 was 7.1. EU's (relative antibody levels) determined for each ELISA plate could then be compared to those of another plate by use of the appropriate multipliers.

G. IgG Anti-Tetanus Toxoid Avidity

A modified ELISA technique was used to determine antibody avidity, based on a report by Pullen et al. (1986). Avidity may be determined by increasing concentrations of chaotropic ions such as ammonium thiocyanate (NH_4SCN), to dissociate antigen-antibody complexes. Theoretically, the strength of antigen-antibody binding (avidity) is proportional to the eluant concentration such that the most avid antibody molecules will require the highest concentrations of eluant to dislodge them from the antigenic epitope.

Immulon polystyrene 96 well microtiter plates were initially coated (200 μl/well) with a solution of 10 mg/ml EDC (1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride [Calbiochem]) in 3 mM phosphate buffered saline containing 0.125 mg/ml poly-L-lysine hydrobromide (type II, mw 240,000 [Sigma Chemical]) (EDC-PLL) which was prepared fresh for each use. Plates were incubated for one hour at room

temperature, aspirated and washed 5 times with PBS to remove excess EDC-PLL and tapped dry. Tetanus toxoid (5 µg/ml) in Sorenson's Buffer was used to coat the plates (200 µl/well), which were then incubated for four hours at 45°C. The plates were washed and tapped dry as before and then allowed to dry further at 37°C. After drying for one hour they were stored at 4°C until used (maximum of 2 weeks). The pretreatment with EDC solution containing poly-L-lysine allowed covalent attachment of antigen (tetanus toxoid) to the plates.

Prior to use, the plates were washed five times with PBS to remove unbound antigen and tapped dry. Serum samples were applied (100 µl/well) to the plates such that equal dilutions of sera from a single monkey representing one collection time point were analyzed in duplicate. In this manner, serum samples from a single time point for all five monkeys were examined on one plate. After 30 minutes incubation at room temperature on a rotator, NH$_4$SCN (0, 0.1, 0.2, 0.5, 1, 2, 4, and 5 Molar) was added to the serum samples in each well. Following incubation for 15 minutes at room temperature, the plates were washed and tapped dry as before to remove unbound sera and eluant. GAHG at a dilution of 1:500 in 0.5% blotto was added (100 µl/well), with subsequent incubation of the plate for 2 hours at room temperature on a rotator. Plates were washed and tapped dry as before to remove unbound GAHG. A 1:800 dilution of RAGG- AP in PBS was then applied to all wells (100 µl/well), and plates were incubated overnight at room temperature on a rotator.

The following day, plates were washed with PBS 5 times, tapped dry, and developed with 1 mg/mL NNP (200 µl/well) for 30 minutes or until sufficient color change. This reaction was then terminated with 1N NaOH (100 µl/well), at which time the extent of color change was determined spectrophotometrically at 410 (Dynatech MR 650). The OD was related to the log$_{10}$ of the molar concentration of NH$_4$SCN and a linear regression analysis calculated. The avidity index (AI) was determined as that molar concentration of NH$_4$SCN which resulted in a 50% reduction in OD when no eluant was added.

H. Statistical Analyses

Significance of the changes in antibody levels over baseline post-primary and post- secondary immunization was determined for total IgG and each IgG subclass by Wilcoxon matched-pairs signed-rank analysis to $p < 0.05$ significance. Significance of change in IgG avidity over baseline post primary and post secondary immunization was determined by Wilcoxon matched-pairs signed-rank analysis to $p < 0.05$ significance. Correlation of change in IgG antibody levels with change in avidity was evaluated using Spearman rank correlation analyses to $p < 0.05$ significance. These test were performed on an IBM PC based system using Statgraphics (STSC) software.

I. Buffers

Sorenson's buffer (pH 5.9)

7.96 g KH_2HPO_4

8.0 g NaCl

1.45 g $NaHPO_4.2H_2O$

bring to 1 liter with deionized water

Stock buffers:

0.2M Sodium carbonate

(21.2 g sodium carbonate/1000 ml distilled water)

0.2M sodium bicarbonate

16.8 g sodium bicarbonate/1000 ml distilled water)

10% Sodium azide (10 g NaN_3/100 ml distilled water)

Phosphate Buffered Saline

10.8g Na_2HPO_4

1.1g Na_2PO_4-H_2O

350g NaCl

bring to 500 ml with distilled water

Buffer I: for coating plates with antigen (pH 9.6)

80 ml sodium bicarbonate stock

170 ml sodium bicarbonate stock

1 ml sodium azide

bring to 500 ml with distilled water

Buffer III: for diluting samples and antisera

300 ml 10X PBS

1.5 ml Tween 20

6 ml sodium azide

bring to 3 L with distilled water

Buffer IV: for dissolving NPP substrate (pH 9.8)

        55 ml sodium carbonate stock

        70 ml sodium bicarbonate stock

        100 mg magnesium chloride

        bring to 500 ml with distilled water

III. RESULTS

A. Development of ELISA for Antibody to Tetanus Toxoid and Validation of the Use of Anti-Human Antibodies for Detection of Monkey Serum Antibody

1. Development of Anti-TT ELISA

Determination of appropriate antigen coating concentration was accomplished by coating wells with 0.5 - 50 µg/well TT on Immulon microtiter plates (Dynatech), blocking for 2 hours with 0.1% gelatin (to reduce nonspecific binding), followed by application of hyperimmune reference serum. The reference serum was applied in serial two fold dilutions beginning at 1:50. Optimal conditions for strong and clear signal relative to background occurred with an antigen coating of 5 µg/well TT and initiating with a serum dilution of 1:100 - 1:200. Similar comparisions were made for the subsequent concentrations of all other reagents, and resulting reagents were used in ELISA systems with NhP serum for these studies.

2. Validation of Anti-human Antibodies for Detection of Monkey Serum Antibody

Once a preliminary system for detection of an anti-tetanus toxoid response was developed in the NhP using anti-human isotype specific antibodies, experiments were performed to characterize and validate the use of these anti-human IgG and IgG subclass reagents.

These preliminary findings indicated that affinity purified goat anti-human IgG was capable of detecting antibody activity to TT in the sera of NhP. The results depicted in **Figure 3** demonstrate that while we cannot directly compare absolute levels of antibody between human and NhP serum, the dynamics of the detection system and the dilutions of serum in which the antibody was detected in the ELISA were generally comparable when comparing the human to the NhP curves. In a crude interpretation, it appeared that the anti-human IgG reagent was capable of detecting NhP antibody at one dilution less than that noted for the human serum. Also, the maximum antibody level noted by this system (i.e. plateau region of

FIGURE 3.

Detection of anti-tetanus toxoid IgG in human and monkey serum. Affinity purified goat anti-human IgG (GAHG) was used to evaluate the reactivity of human serum containing antibody to TT and NhP serum from TT immunized monkeys. Hu/anti-TT denotes the reaction of human anti-TT serum, Hu/norm denotes the reaction of a human serum with low anti-TT IgG levels, Mo/anti-TT denotes the reaction of immune NhP serum, and Mo/norm denotes the reaction of nonimmune NhP serum. The points denote triplicate determinations at each dilution of the experimental sera.

OD 405nm

Log Reciprocal Serum Dilution

Hu/anti-TT   Hu/norm   Mo/anti-TT   Mo/norm
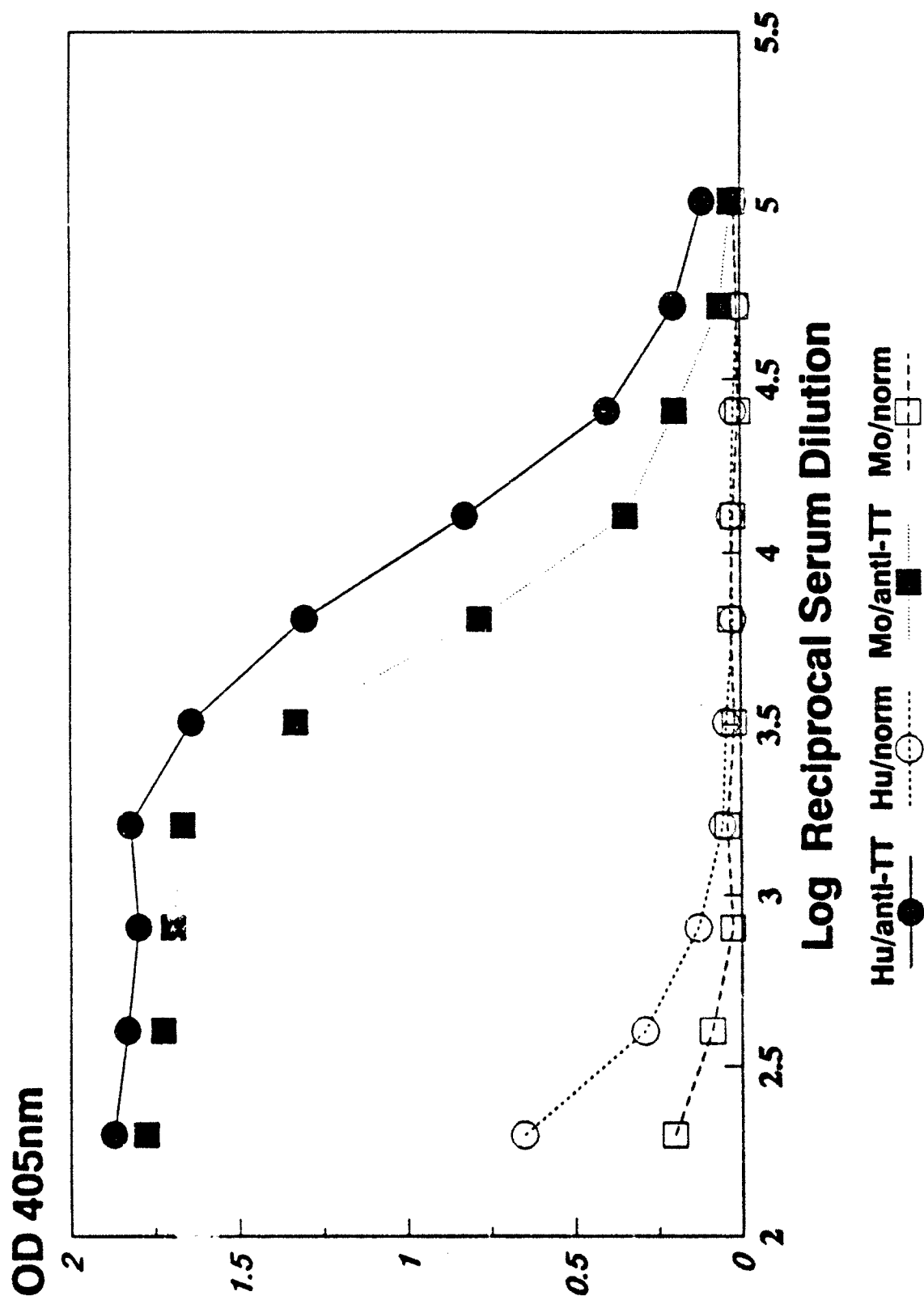
curve) was similar for the two species. Consequently, it appears as though, the anti-human IgG reagent can efficiently detect the presence and quantity of anti-TT antibody in the NhP sera.

Preliminary findings from other experiments had suggested that mouse monoclonal antibodies that were prepared to human IgG subclasses also reacted with molecules in the serum of cynomolgus monkeys. While identification of exact homology between the molecular species in human and nonhuman primate awaits purification of each subclass from the NhP serum, studies were performed to support the similarity in reactivities between the human and nonhuman primate serum components. An initial set of studies were designed to examine the specificity of reactivity of the mouse monoclonal antibodies for each of human IgG subclass proteins. In these cases, ELISA plates were coated with varying amounts of human IgG1, IgG2, IgG3 or IgG4 myeloma proteins. As shown in **Figures 4-7**, each of the monoclonal antibodies was exquisitely specific for the homologous human myeloma protein. Additionally, a competitive inhibition analysis was also developed using both human and nonhuman primate serum. The human serum was used at a dilution containing a predetermined amount of each subclass (i.e. 6-8 µg). The human serum was co-incubated with the mouse monoclonal to a particular IgG subclass in wells coated with the homologous subclass myeloma protein. The results demonstrated a substantial inhibition of the monoclonal binding. A similar dilution of nonhuman primate serum was also used in a competitive assay. In every case the nonhuman primate serum was less inhibitory at a similar dilution as the human serum. The largest difference in inhibitory capacity was noted with IgG1 and IgG2 subclasses. There is no direct method for determining an equal level of subclasses between the two species sera. However, it could be suggested from this data that IgG3 and IgG4 inhibiting ability were more similar due to greater cross-reactivity in antigen epitopes between the species.

A second group of experiments were performed utilizing human and nonhuman primate sera that contained antibody activity to tetanus toxoid. In these studies, ELISA plates were coated with TT and serial dilutions of the human and

FIGURE 4.

Specificity of mouse monoclonal antibody to human IgG1. Anti-IgG1, 2, 3 & 4 denote reactivity of MoAb with each specificity to plates coated with human IgG1 myeloma protein. Hu/ser denotes coincubation of the mouse anti-IgG1 monoclonal with a human serum (0.1 ml at a 1:100 dilution) containing 8.63 µg of IgG1. Mo/ser denotes coincubation of the mouse anti-IgG1 monoclonal with a 1:100 dilution of a monkey serum. The points denote the results of triplicate determinations at each IgG1 coating concentration.

OD 405 nm
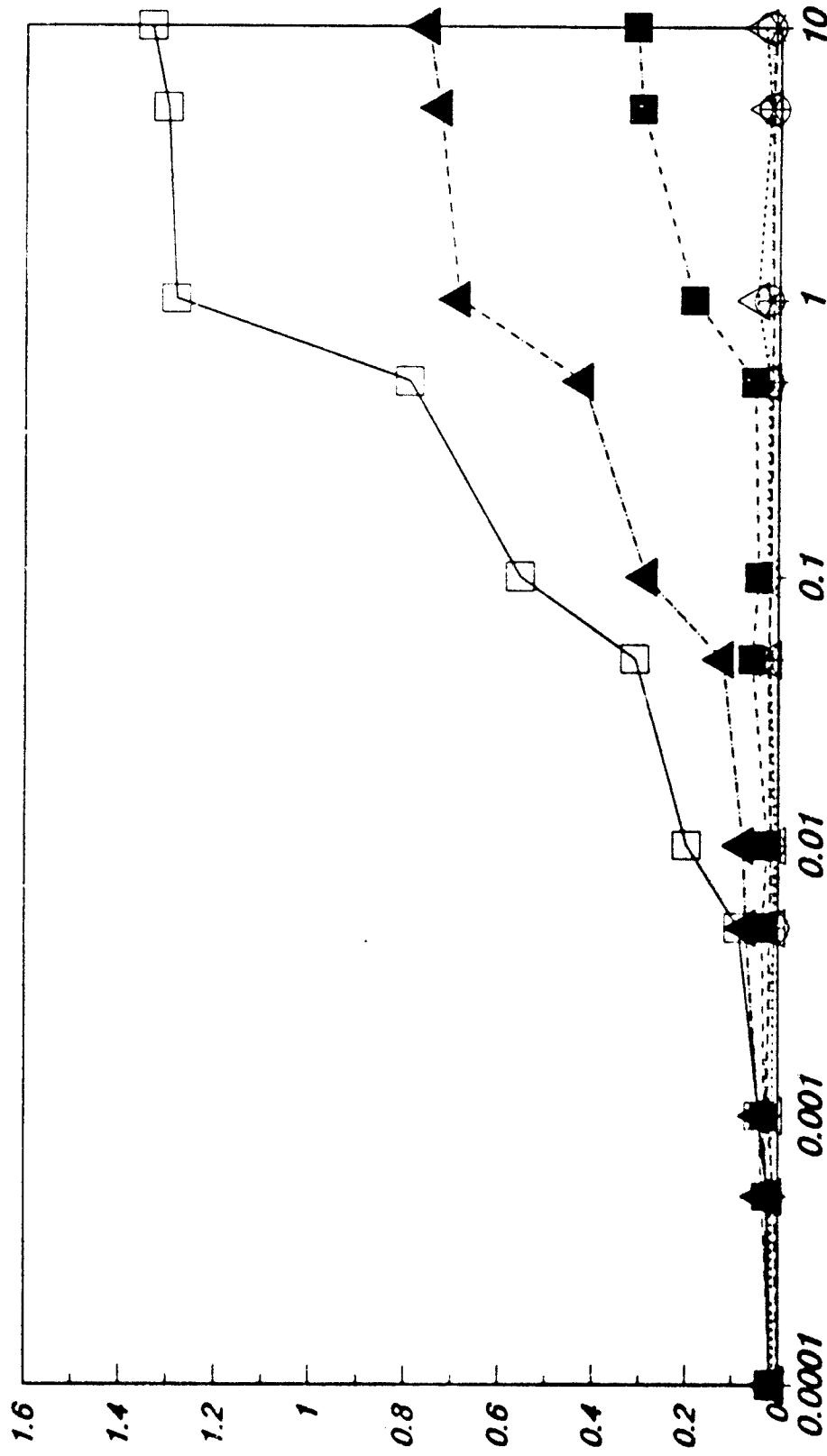
IgG1 Conc. (ug)

anti-IgG1   anti-IgG2   anti-IgG3   anti-IgG4   hu/ser   mo/ser

FIGURE 5.

Specificity of mouse monoclonal antibody to human IgG2. Anti-IgG1, 2, 3 & 4 denote reactivity of McAb with each specificity to plates coated with human IgG2 myeloma protein. Hu/ser denotes coincubation of the mouse anti-IgG2 monoclonal with a human serum (0.1 ml at a 1:50 dilution) containing 7.92 μg of IgG2. Mo/ser denotes coincubation of the mouse anti-IgG2 monoclonal with a 1:50 dilution of a monkey serum. The points denote the results of triplicate determinations at each IgG2 coating concentration.

FIGURE 6.

Specificity of mouse monoclonal antibody to human IgG3. Anti-IgG1, 2, 3 & 4 denote reactivity of MoAb with each specificity to plates coated with human IgG3 myeloma protein. Hu/ser denotes coincubation of the mouse anti-IgG3 monoclonal with a human serum (0.1 ml at a 1:5 dilution) containing 8.54 µg of IgG3. Mo/ser denotes coincubation of the mouse anti-IgG3 monoclonal with a 1:5 dilution of a monkey serum. The points denote the results of triplicate determinations at each IgG3 coating concentration.

OD 405 nm

IgG3 Conc. (ug)

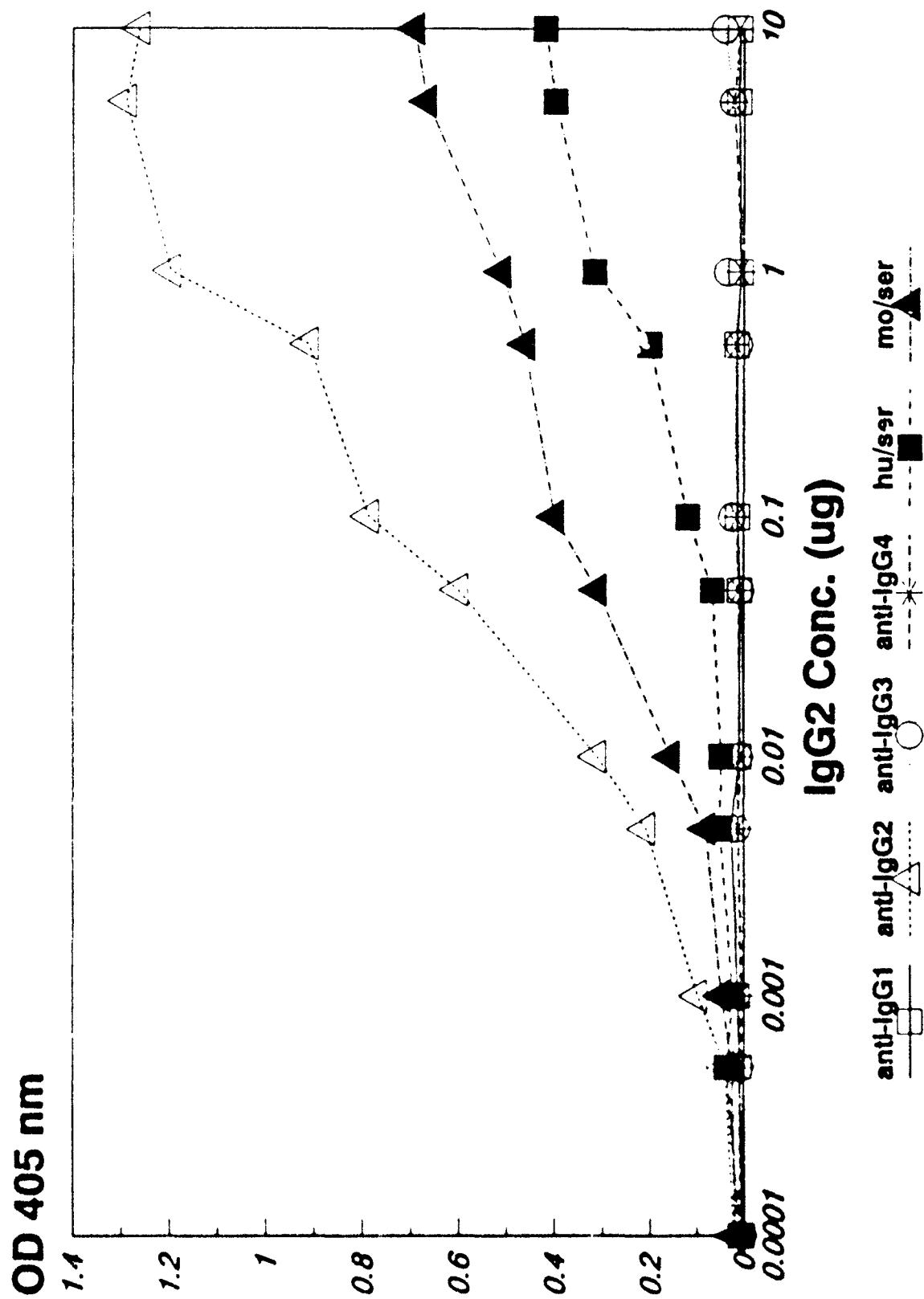anti-IgG1   anti-IgG2   anti-IgG3   anti-IgG4   hu/ser   mo/ser

45

FIGURE 7.

Specificity of mouse monoclonal antibody to human IgG4. Anti-IgG1, 2, 3 & 4 denote reactivity of MoAb with each specificity to plates coated with human IgG4 myeloma protein. Hu/ser denotes coincubation of the mouse anti-IgG4 monoclonal with a human serum (0.1 ml at a 1:5 dilution) containing 6.08 µg of IgG4. Mo/ser denotes coincubation of the mouse anti-IgG4 monoclonal with a 1:5 dilution of a monkey serum. The points denote the results of triplicate determinations at each IgG4 coating concentration.
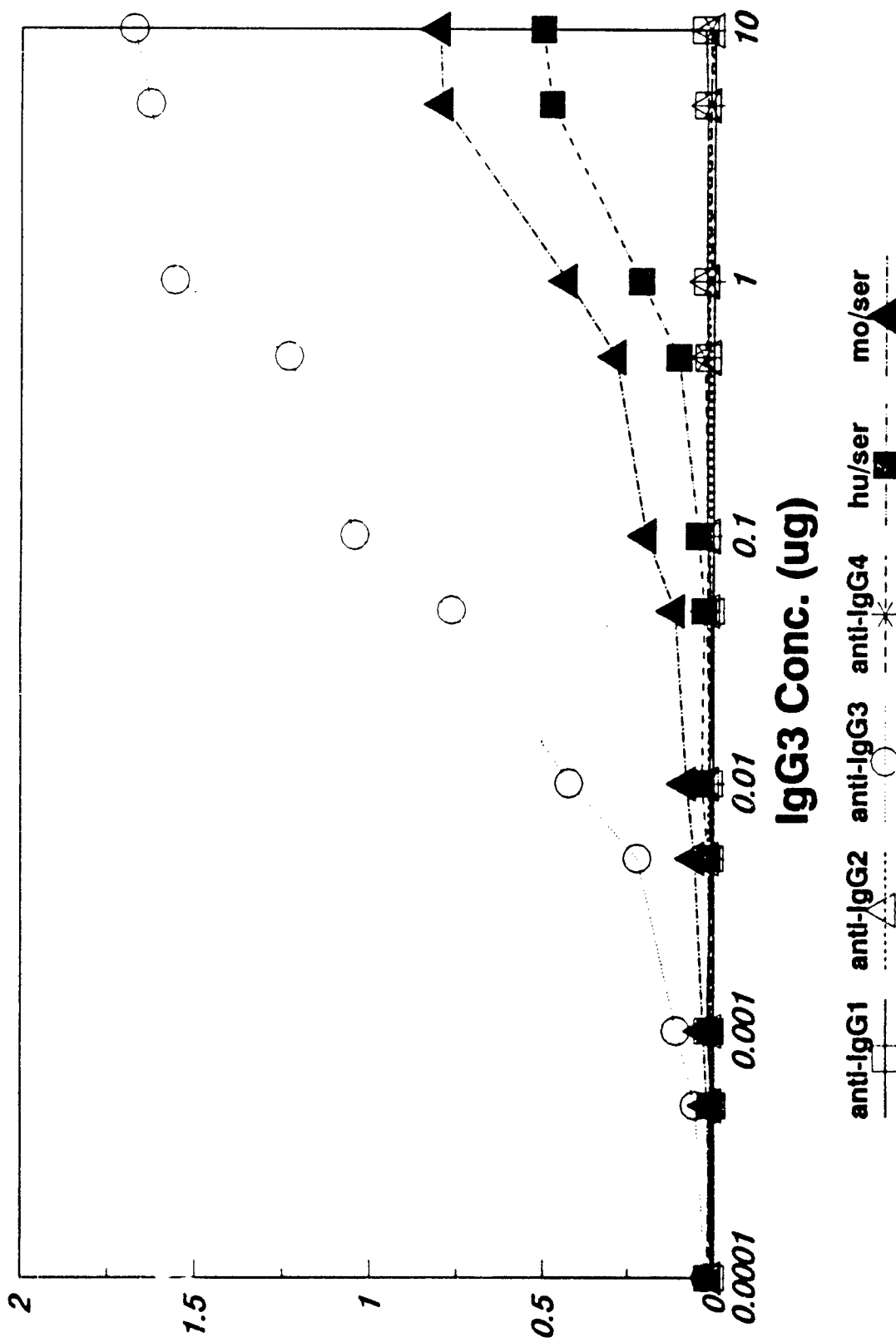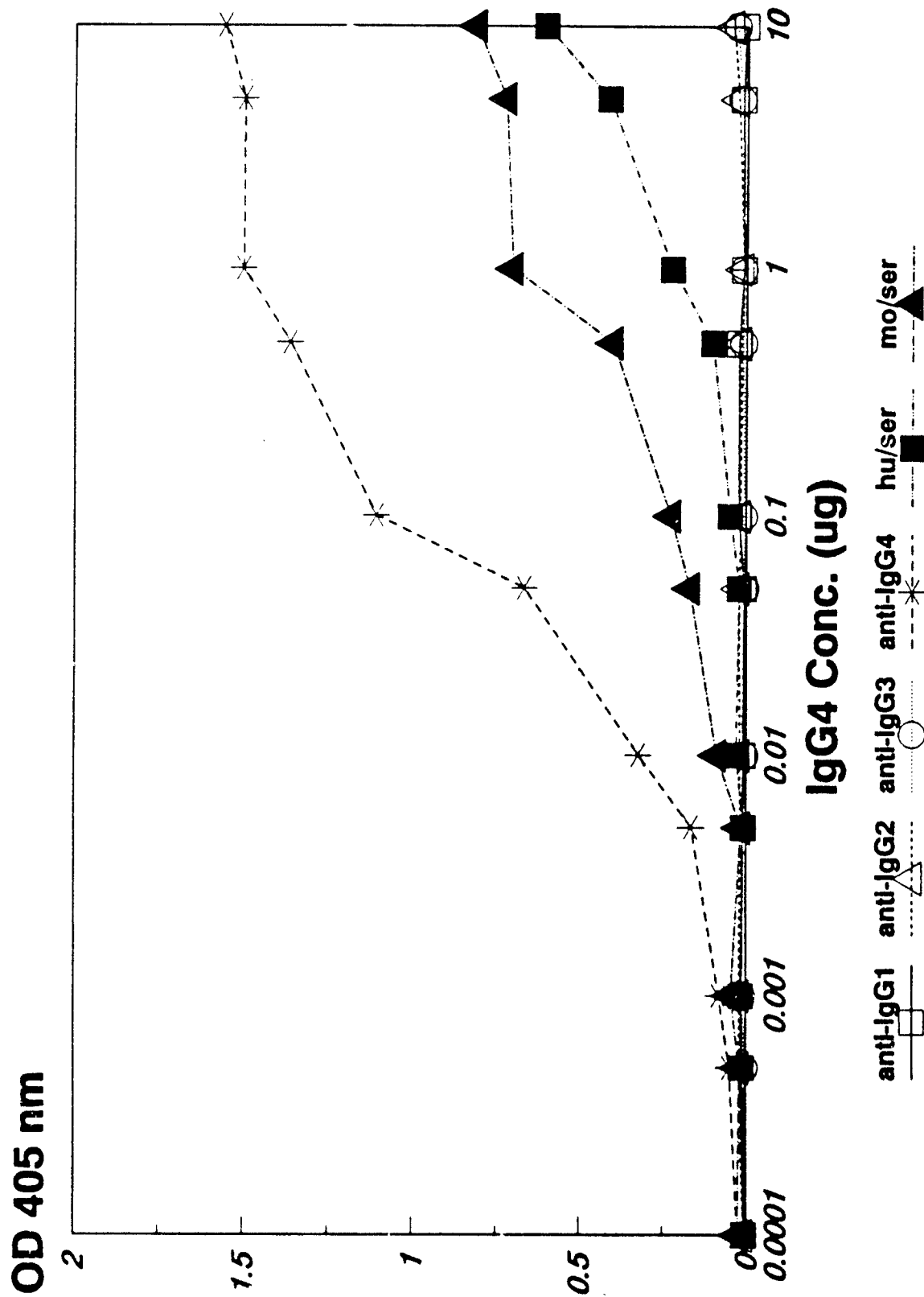
OD 405 nm

IgG4 Conc. (ug)

anti-IgG1   anti-IgG2   anti-IgG3   anti-IgG4   hu/ser   mo/ser

nonhuman sera were incubated and antibody reactivity developed with IgG subclass specific monoclonal antibodies (**Figures 8-11**). In each case, the reactivity in the human serum was greater, presumably resulting from more efficient interaction of the monoclonals with the homologous human IgG molecules. However, the primary antibody response to this protein antigen in both species was of the IgG1 and IgG3 subclasses. Human myeloma proteins that were homologous to the monoclonal antibody specificities were added to other wells (100 ng/well) in a competitive assay with the monoclonal antibody after human or nonhuman primate antibody had bound to the TT antigen. With each subclass the characteristics of inhibition of binding to the human and nonhuman primate antibody were similar, suggesting that comparable antibody molecules were being detected in the two species serum.

Finally, a group of studies was performed in which cross-competitive inhibitions were examined between human and nonhuman primate serum immunoglobulins. Specifically, ELISA plates were coated with TT and incubated with either human or nonhuman primate serum containing antibody to TT. The system was then developed with subclass specific monoclonal antibody. A competitive inhibition was created by incubating either nonhuman primate serum lacking anti-TT or with a human serum containing a low level of IgG antibody to TT. As shown in **Table 1**, the competition between human serum molecules, as well as competitive inhibition of nonhuman primate immunoglobulin molecules demonstrated a nearly complete inhibition. The ability of the human serum IgG to block the nonhuman primate IgG reactivity was uniformly greater for each subclass than was the reverse competition. Although, in each case, the evidence indicates a substantial antigenic relationship (45-85%) between molecules in the human and NhP sera that have antibody activity to TT. Thus, while these studies cannot definitively state that there are identical IgG subclass molecules in the serum from these two species, each experiment provided more compelling evidence for an identity of IgG subclasses between humans and cynomolgus monkeys.

FIGURE 8.

Specificity of mouse monoclonal antibody to human IgG1 for detection of human and NhP antibody to TT. The ELISA plates were coated with TT as antigen an a human serum containing high levels of anti-TT antibody, as well as immunized NhP sera were tested for antibody to TT. Hu/ser denotes the reactivity of the human serum as detected by incubation with the anti-IgG1 MoAb. Hu/G1 denotes coincubation of the mouse anti-IgG1 monoclonal with 100 ng of a human IgG1 myeloma protein. Mo/ser denotes the reactivity of the NhP serum as detected by incubation with the anti-IgG1 MoAb. Mo/G1 denotes coincubation of the mouse anti-IgG1 monoclonal with 100 ng of a human IgG1 myeloma protein. The points denote the results of triplicate determinations at each dilution of the experimental sera.

OD 405 nm

Log$_{10}$ Reciprocal Serum Dilution

hu/ser ● mo/ser ■ hu/G1 ○ mo/G1 □
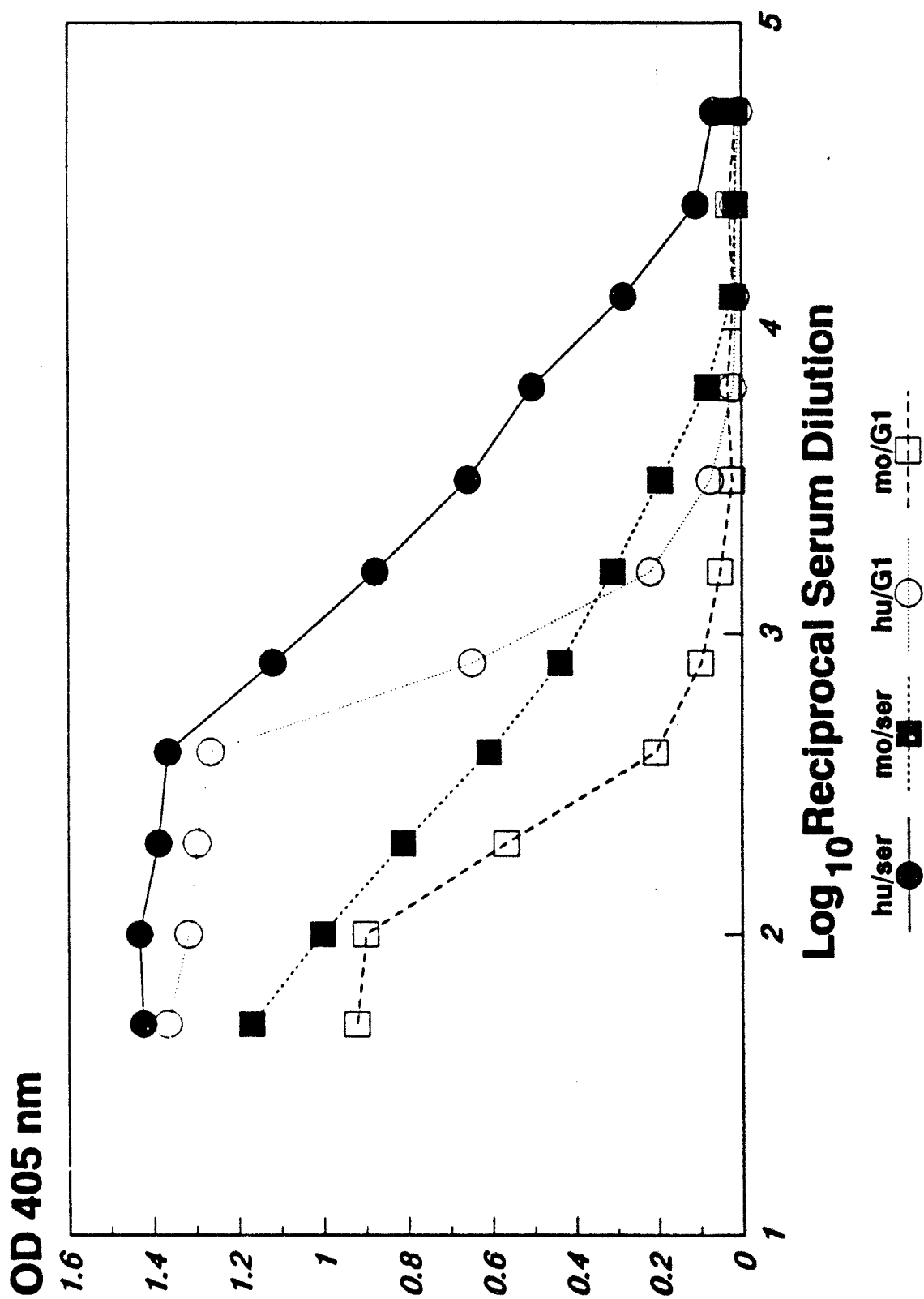
FIGURE 9.

Specificity of mouse monoclonal antibody to human IgG2 for detection of human and NhP antibody to TT. The ELISA plates were coated with TT as antigen an a human serum containing high levels of anti-TT antibody, as well as immunized NhP sera were tested for antibody to TT. Hu/ser denotes the reactivity of the human serum as detected by incubation with the anti-IgG2 MoAb. Hu/G2 denotes coincubation of the mouse anti-IgG2 monoclonal with 100 ng of a human IgG2 my oma protein. Mo/ser denotes the reactivity of the NhP serum as detected by incubation wi h the anti-IgG1 MoAb. Mo/G2 denotes coincubation of the mouse anti-IgG2 monoclonal with 100 ng of a human IgG2 myeloma protein. The points denote the results of triplicate determinations at each dilution of the experimental sera.
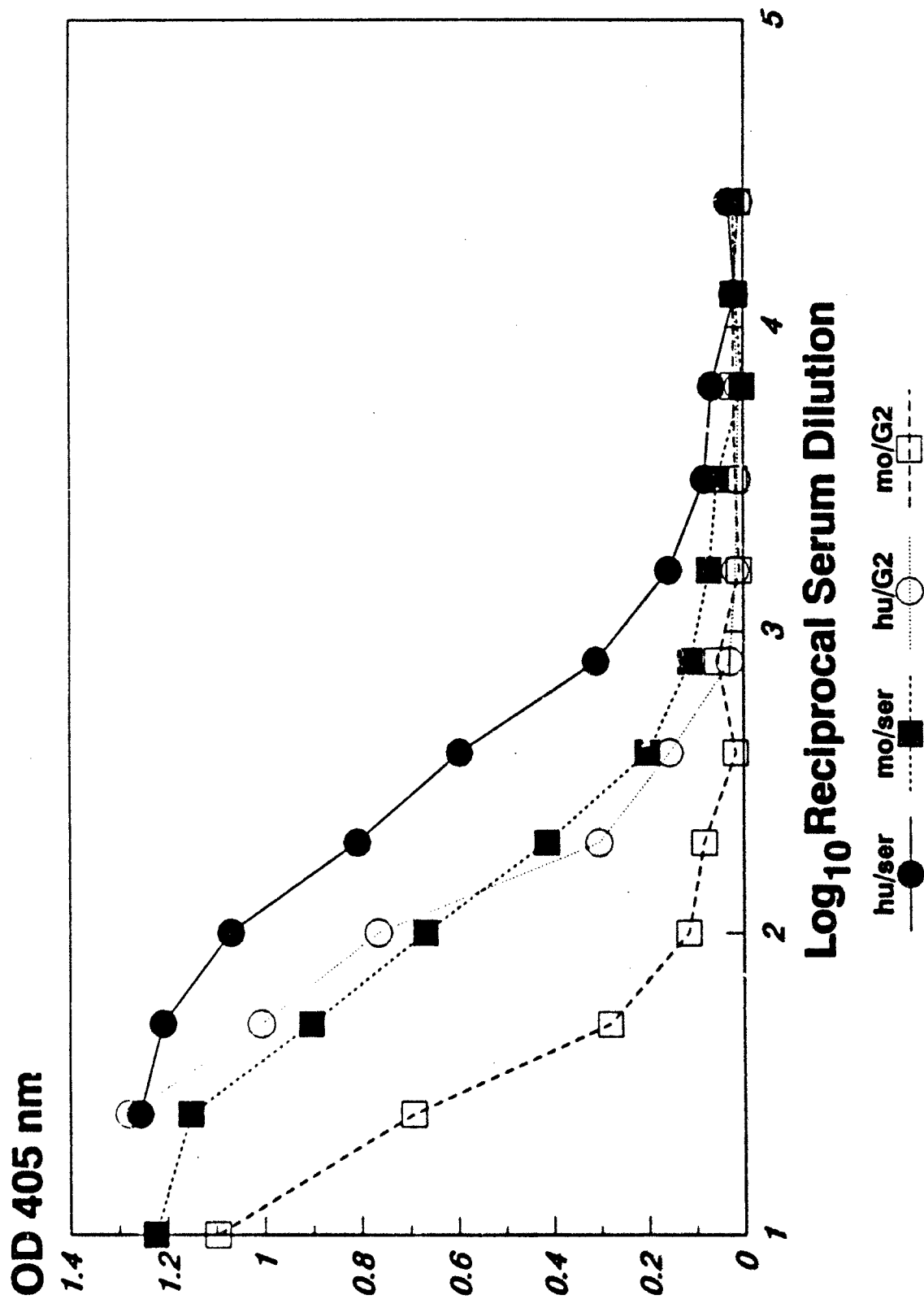
FIGURE 10.

Specificity of mouse monoclonal antibody to human IgG3 for detection of human and NhP antibody to TT. The ELISA plates were coated with TT as antigen an a human serum containing high levels of anti-TT antibody, as well as immunized NhP sera were tested for antibody to TT. Hu/ser denotes the reactivity of the human serum as detected by incubation with the anti-IgG3 MoAb. Hu/G3 denotes coincubation of the mouse anti-IgG3 monoclonal with 100 ng of a human IgG3 myeloma protein. Mo/ser denotes the reactivity of the NhP serum as detected by incubation with the anti-IgG3 MoAb. Mo/G3 denotes coincubation of the mouse anti-IgG3 monoclonal with 100 ng of a human IgG3 myeloma protein. The points denote the results of triplicate determinations at each dilution of the experimental sera.

OD 405 nm
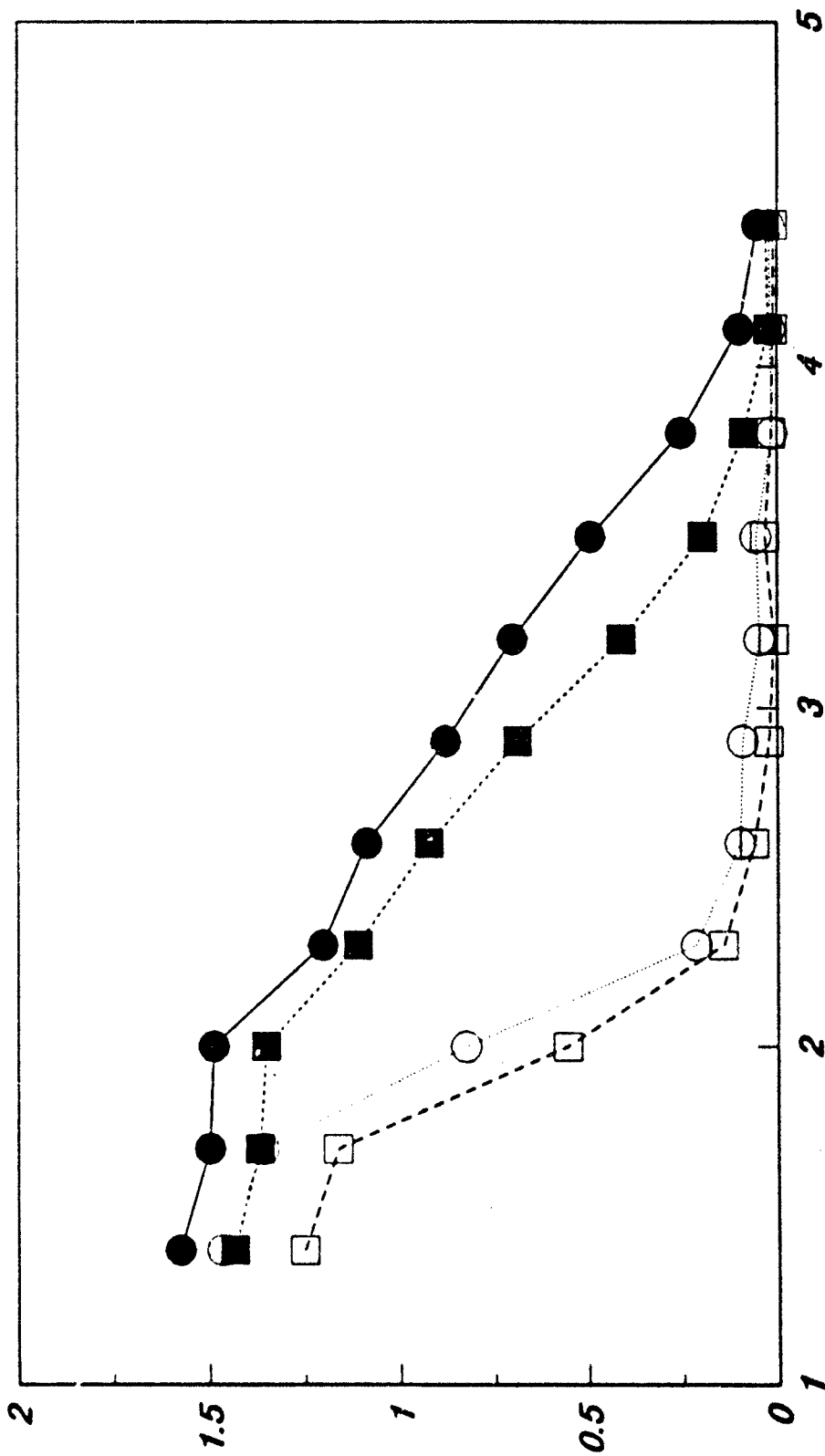
Log$_{10}$Reciprocal Serum Dilution

hu/ser ●   mo/ser ■   hu/G3 ○   mo/G3 □

FIGURE 11.

Specificity of mouse monoclonal antibody to human IgG4 for detection of human and NhP antibody to TT. The ELISA plates were coated with TT as antigen an a human serum containing high levels of anti-TT antibody, as well as immunized NhP sera were tested for antibody to TT. Hu/ser denotes the reactivity of the human serum as detected by incubation with the anti-IgG4 MoAb. Hu/G4 denotes coincubation of the mouse anti-IgG4 monoclonal with 100 ng of a human IgG4 myeloma protein. Mo/ser denotes the reactivity of the NhP serum as detected by incubation with the anti-IgG4 MoAb. Mo/G4 denotes coincubation of the mouse anti-IgG4 monoclonal with 100 ng of a human IgG4 myeloma protein. The points denote the results of triplicate determinations at each dilution of the experimental sera.
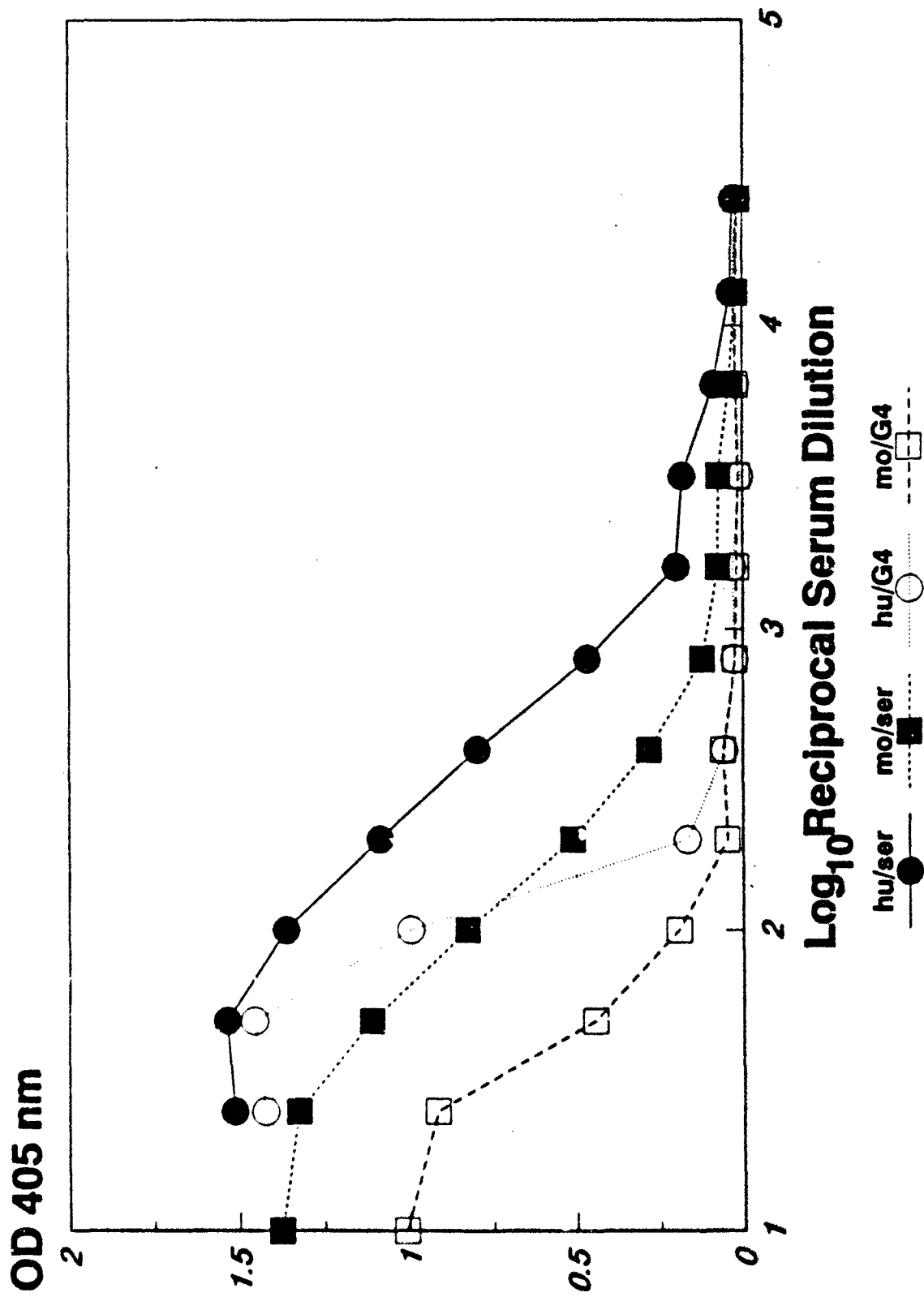
.ABLE 1

Antigenic Relationship of I_gG Subclasses in Human and Nonhuman Primate Sera

Antisera	MoAb Specificity	Inhibiting Sera	% Antibody Inhibition
Human Anti-TT	IgG1	Human NhP	87[a] 45
Human Anti-TT	IgG2	Human NhP	96 63
Human Anti-TT	IgG3	Human NhP	94 85
Human Anti-TT	IgG4	Human NhP	81 69
NhP Anti-TT	IgG1	Human NhP	78 88
NhP Anti-TT	IgG2	Human NhP	68 90
NhP Anti-TT	IgG3	Human NhP	93 93
NhP Anti-TT	IgG4	Human NhP	74 85

[a] denotes mean % of antibody activity inhibited by incubation of murine monoclonal antibody simultaneously with the inhibiting sera. Generally, the variance of triplicate determinations was 3-7% of the mean.

B. IgG Antibody Responses to TT Immunization

The protocol for examination of NhP immune responses to a protein toxin was described earlier in **Figure 1**. IgG antibody response for each monkey is shown in **Figure 12 and Table 2**. As expected, no IgG antibody activity was apparent up to one week post-primary immunization. After this interval, a significant increase in antibody activity of approximately 40 fold was detected at 1 month ($p < 0.007$). Maximum anti-TT IgG serum levels (110 fold over baseline) were attained by one week to one month post-secondary immunization. This change was significantly greater than baseline ($p < 0.007$) and to levels one month post primary ($p < 0.005$). A plateau in response was then apparent until about two months post-secondary immunization, at which time the antibody activity decreased dramatically (~50% decrease/month). All 5 monkeys reflected this general response pattern exhibiting increases in activity until 7-30 days post secondary immunization, and all were in decline between 2-3 months post-secondary immunization.

Individual monkeys showed broad variability in response rate and maximal anti-TT IgG levels. Absolute levels of anti-TT IgG at maximum response varied from 99-292 EU, while the extent of change was distributed between 75-430 fold increase over baseline levels. Although NhP M99 mounted the greatest response in absolute levels of total anti-TT IgG (292 EU), the greatest extent of change from baseline (430 fold increase) occurred in P69 which exhibited the lowest absolute response (99 EU).

C. Characterization of IgG Subclass Response to Tetanus Toxoid in the NhP

Previous investigations have reported some restriction of IgG subclass responses to various antigenic chemical structures in humans (Hammarstrom & Smith 1986). In the normal adult, IgG responses to protein antigens are usually restricted to IgG1 subclass with minor contributions from IgG3 and IgG4, whereas polysaccharide antigens evoke a response dominated by IgG2. IgG4 has been associated more with chronic antigenic stimulation.

1. IgG1 Response

Primary immunization resulted in an average 48 fold increase in IgG1 levels at 1 month which further increased an average of 133 fold over

FIGURE 12.

IgG response in each of the non-human primates (NhP) after primary and secondary immunization with the protein tetanus toxoid. Development of the relative antibody levels as measured in ELISA Units over time. The lines represent the response development in M79 □, M99 +, P61 ◇, P69△, R25 ✕, and ▽ portrays the average response for all five monkeys. The points represent the mean value of triplicate determinations at each time interval.

TOTAL IgG RESPONSE IN EACH NhP

TABLE 2

IgG Response in the Nonhuman Primate to Immunization
with Tetanus Toxoid

NhP	BASE	PRIMARY		SECONDARY					
		7	30	3	7	30	60	90	120
M79	2.63[a]	2.68	106.56	114.20	197.12	190.58	NT	NT	NT
M99	1.96	1.66	120.99	238.05	255.20	292.59	287.22	168.34	93.00
P61	1.84	2.23	21.33	34.65	141.14	111.97	105.78	24.78	5.45
P69	0.24	0.27	19.29	37.89	81.76	99.13	89.15	69.59	16.86
R25	1.11	0.95	38.90	52.69	180.75	118.96	128.65	68.16	30.07
MEAN	1.55	1.56	61.41	95.50	171.19	162.65	152.70	82.72	36.33
SD	0.82	0.86	43.53	76.85	57.83	72.36	78.92	52.61	33.86

[a] denotes mean IgG antibody level expressed as ELISA Units (EU).
NT denotes not tested due to death of the animal unrelated to this study.

baseline after secondary immunization and which were both significantly elevated when compared to baseline levels (p < 0.02)(**Figure 13 and Table 3**). Individual animals responded as high as 450 fold over baseline at maximal response. Some variation in time of maximum response post secondary immunization was noted in that two animals peaked at one week after the booster immunization while the remaining three peaked at one month post immunization.

2. IgG2 Response

Primary immunization resulted in an average 25 fold increase in IgG2 levels at 1 month which further increased an average of 131 fold over baseline at maximal response (3 months post secondary) which were both significant over baseline levels (p < 0.02)(**Figure 14 and Table 4**). However, baseline levels were, on average very low which explains the low absolute levels at maximal response while exhibiting a large increase over baseline. Individual animals responded as high as 391 fold over baseline at maximal response. While there appeared to be a generalized early response to secondary immunization at three days to one week post-secondary immunization, two animals uniquely demonstrated a peak response in the IgG2 subclass at 3 months post-secondary immunization. This was at a time when all other subclasses were in decline.

3. IgG3 Response

Primary immunization resulted in an average 35 fold increase in IgG3 levels at 1 month which further increased an average of 86 fold over baseline at maximal response (1 week post-secondary) which were both significantly greater than baseline levels (p < 0.02) (**Figure 15 and Table 5**). Individual animals responded as high as 465 fold over baseline at maximal response. Interestingly, the time of IgG3 peak response was different for each monkey, and varied from one month post primary immunization in M79 to 2 months post secondary in R25.

4. IgG4 Response

Primary immunization resulted in an average 24 fold increase in IgG4 levels at 1 month which further increased an average of 206 fold over baseline at maximal response (3 month post secondary) which were both significant when compared to

FIGURE 13.

IgG1 response in each of the non-human primates (NhP) after primary and secondary immunization with the protein tetanus toxoid. Development of the relative antibody levels as measured in ELISA Units over time. The lines represent the response development in M79 □, M99 +, P61 ◇, P69 △, R25 ✕, and ▽ portrays the average response for all five monkeys. The points represent the mean value of triplicate determinations at each time interval.
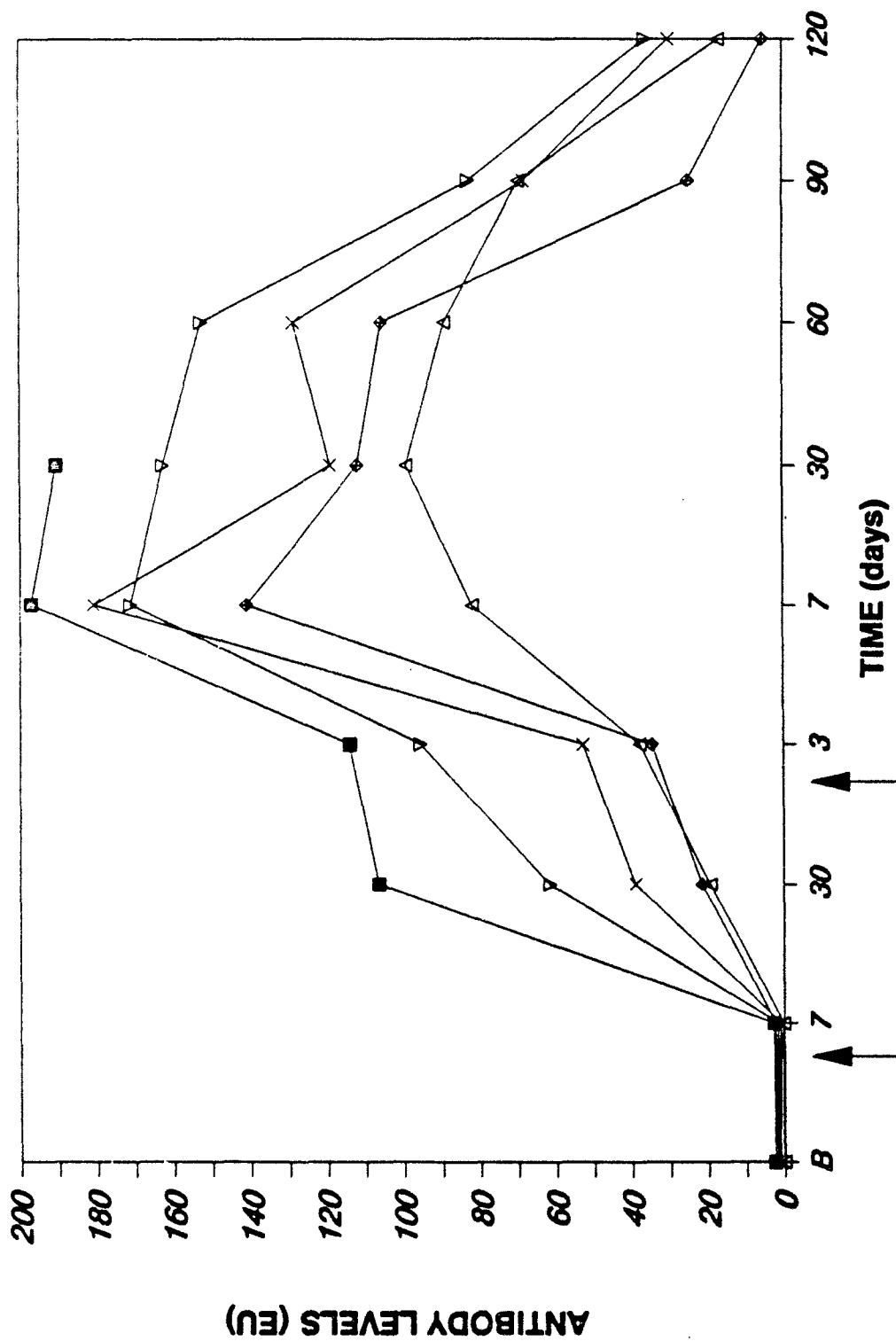
IgG1 RESPONSE IN NhP

ANTIBODY LEVELS (EU)

TIME (days)

TABLE 3

IgG1 Response in the Nonhuman Primate to Immunization
with Tetanus Toxoid

NhP	BASE	PRIMARY		SECONDARY					
		7	30	3	7	30	60	90	120
M79	1.21[a]	1.84	66.48	83.70	147.54	150.32	NT	NT	NT
M99	1.03	0.86	81.79	127.60	141.26	195.81	174.14	65.62	40.80
P61	0.99	1.22	13.14	20.62	76.48	63.82	70.32	13.27	3.53
P69	0.12	0.15	10.55	21.10	37.72	54.42	47.38	33.00	7.37
R25	0.58	0.43	18.47	27.20	100.73	62.65	45.62	20.33	8.87
MEAN	1.79	0.90	38.09	56.04	100.74	105.41	84.36	33.05	8.87
SD	0.39	0.60	29.94	42.88	40.97	57.18	52.74	20.08	14.94

[a] denotes mean IgG antibody level expressed as ELISA Units (EU).
NT denotes not tested due to death of the animal unrelated to this study.

FIGURE 14.

IgG2 response in each of the non-human primates (NhP) after primary and secondary immunization with the protein tetanus toxoid. Development of the relative antibody levels as measured in ELISA Units over time. The lines represent the response development in M79 □, M99 +, P61 ◇, P69 △ , R25 ✕ , and ▽ portrays the average response for all five monkeys. The points represent the mean value of triplicate determinations at each time interval.
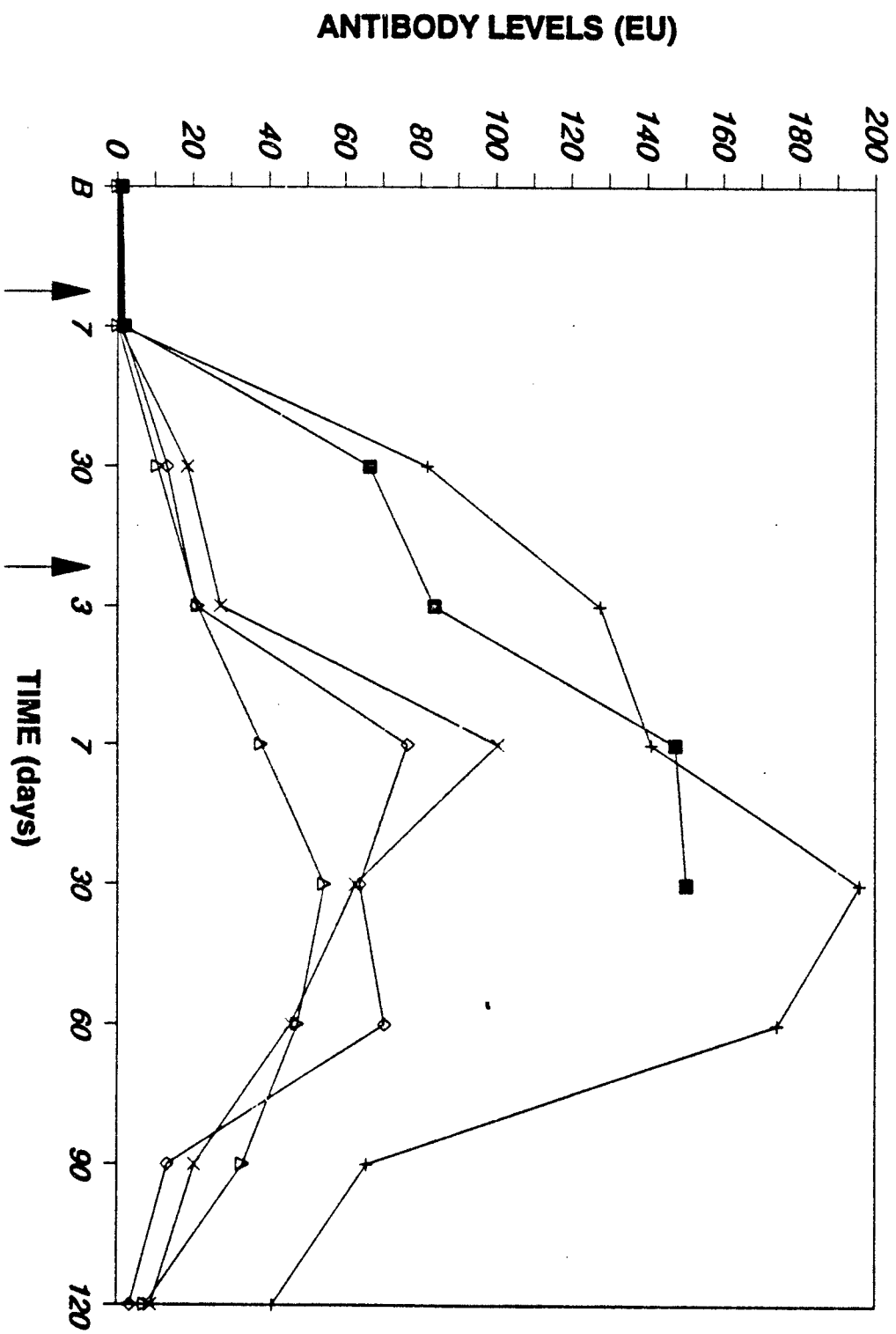
IgG2 RESPONSE IN NhP

ANTIBODY LEVELS (EU)

TIME (days)

TABLE 4

IgG2 Response in the Nonhuman Primate to Immunization with Tetanus Toxoid

NhP	BASE	PRIMARY		SECONDARY					
		7	30	3	7	30	60	90	120
M79	0.16[a]	0.18	4.29	2.70	27.56	18.29	NT	NT	NT
M99	0.12	0.13	6.91	19.93	19.91	10.91	4.78	51.83	6.59
P61	0.39	0.31	1.67	3.81	15.15	7.54	5.57	5.24	0.55
P69	0.02	0.02	1.75	2.66	7.38	2.60	5.43	6.17	1.18
R25	0.07	0.11	4.07	4.49	6.52	7.08	6.73	15.09	5.33
MEAN	0.15	0.15	3.74	6.72	15.39	9.28	5.63	19.58	3.41
SD	0.13	0.09	1.93	6.64	7.80	5.22	0.71	19.01	2.60

[a] denotes mean IgG antibody level expressed as ELISA Units (EU).
NT denotes not tested due to death of the animal unrelated to this study.

FIGURE 15.

IgG3 response in each of the non-human primates (NhP) after primary and secondary immunization with the protein tetanus toxoid. Development of the relative antibody levels as measured in ELISA Units over time. The lines represent the response development in M79 □, M99 +, P61 ◇, P69 △, R25 ✕, and ▽ portrays the average response for all five monkeys. The points represent the mean value of triplicate determinations at each time interval.
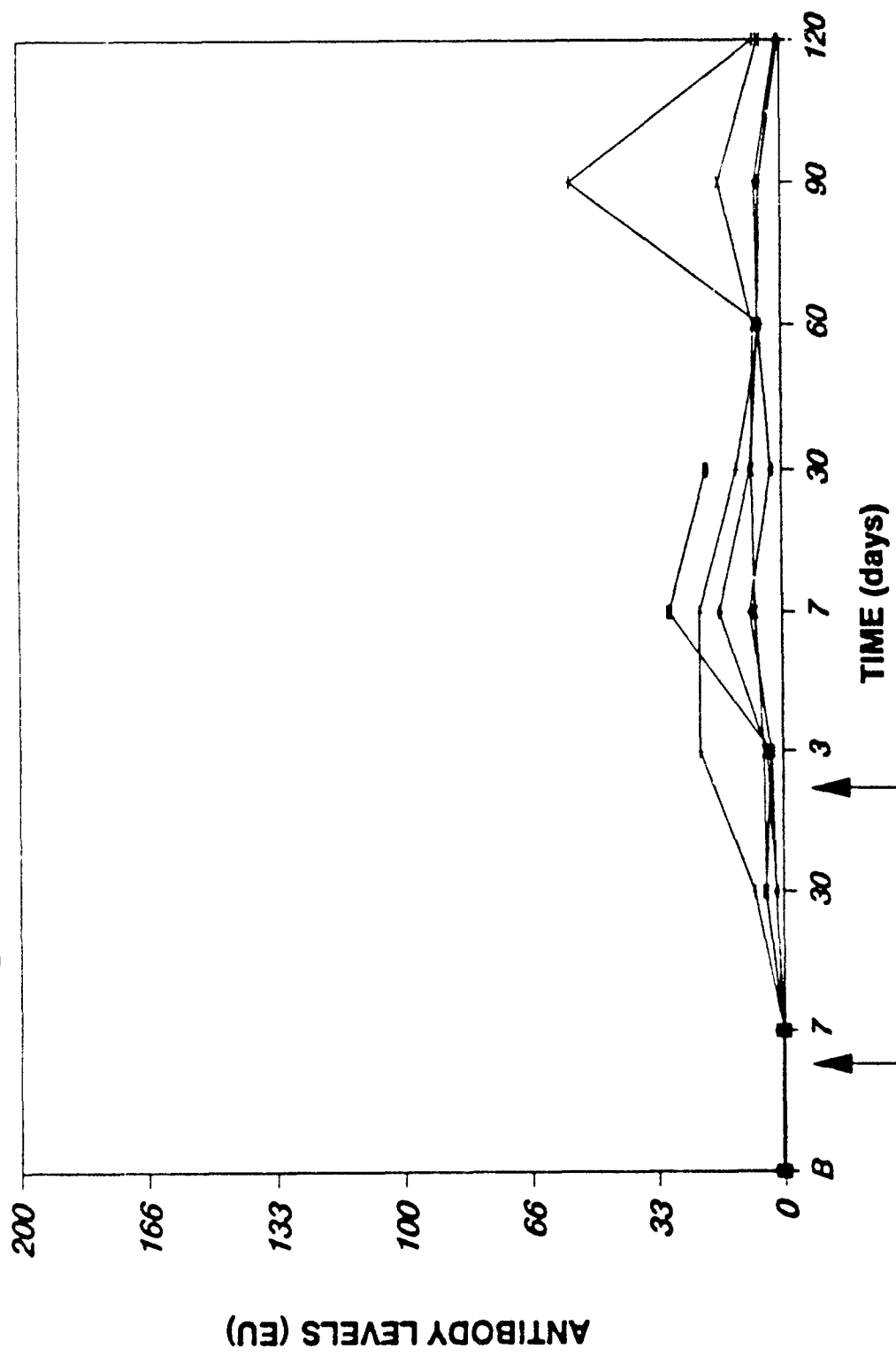
IgG3 RESPONSE IN NhP

ANTIBODY LEVELS (EU)

TIME (days)

TABLE 5

IgG3 Response in the Nonhuman Primate to Immunization
with Tetanus Toxoid

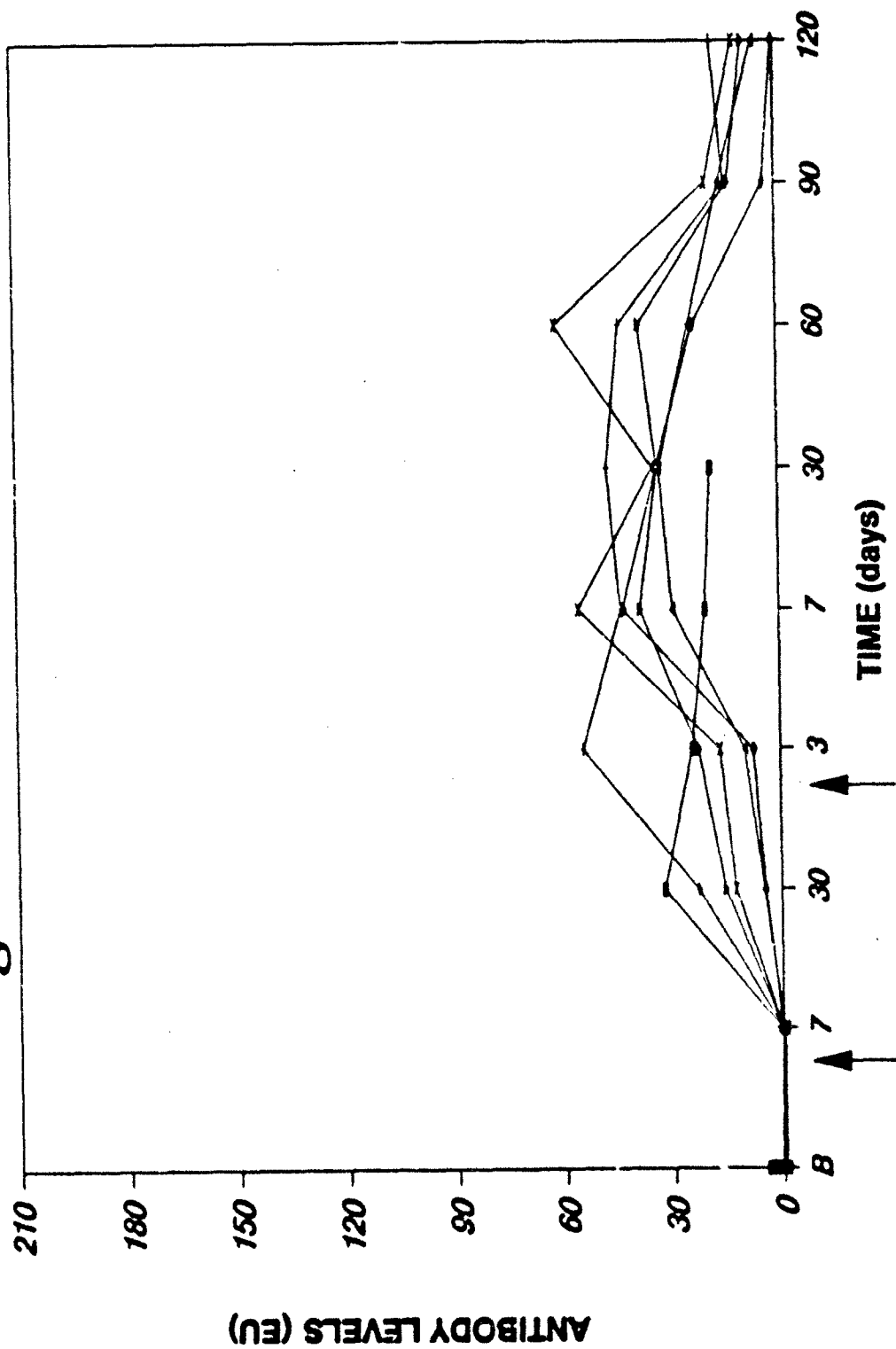| NhP	BASE	PRIMARY		SECONDARY					
		7	30	3	7	30	60	90	120
M79	1.00[a]	0.52	32.20	24.35	20.65	18.78	NT	NT	NT
M99	0.43	0.40	22.84	54.09	43.41	46.90	43.38	14.13	17.65
P61	0.33	0.53	4.89	7.69	42.80	33.47	23.24	3.79	0.91
P69	0.07	0.07	5.17	10.13	29.38	32.64	24.29	15.79	6.28
R25	0.38	0.35	12.90	16.89	55.12	34.65	60.81	19.37	11.73
MEAN	0.44	0.37	15.60	22.63	38.27	33.29	37.93	13.27	9.14
SD	0.31	0.17	10.57	16.76	12.00	8.92	15.45	5.79	6.23

[a] denotes mean IgG antibody level expressed as ELISA Units (EU).
NT denotes not tested due to death of the animal unrelated to this study.

baseline levels (p < 0.02)(**Figure 16 and Table 6**). Individual animals responded as high as 488 fold over baseline at maximal response. Absolute levels of antibody produced by the IgG4 response were actually quite low in all monkeys except one. M99 demonstrated levels at peak response and throughout development of the response that ranged from two to eight times that of the next most responsive NhP.

5. Subclass Participation in Total IgG Response

Determination of subclass participation in the overall IgG response was based on comparison of maximum OD attainable when the various monoclonal IgG subclass detection antibodies were titrated against a static level of NhP reference serum. This comparison is justified based on the following assumptions: 1) The monoclonal antibodies used to detect each IgG subclass are very specific and are directed, most likely, against a single epitope on each NhP IgG subclass molecule. 2) All of the monoclonal antibodies were raised in Balb/c mice, and therefore display similar epitopes themselves across all subclasses. This would support the concept that the affinity purified goat anti-mouse polyclonal serum which is subsequently directed against the monoclonal antibodies will detect, if not a single epitope on the mouse monoclonal, at least similar epitopes across all subclasses. 3) If assumptions 1 and 2 above are correct, then for each NhP anti-TT subclass molecule attached to the plate, an equivalent amount of detection monoclonals and subsequent reagents will be piggy-backed with development of the ELISA. The optical densities of each of the individual subclasses, then, would be proportional to the amount of NhP IgG1, IgG2, IgG3, or IgG4 anti-TT attached to the plate. Since this subclass proportional determination was accomplished with reference serum, which was present on all ELISA plates during IgG subclass studies, comparisons can be made between NhP serum samples on all plates.

Estimation of the relative contribution of each IgG subclass to the total IgG antibody response in reference sera allowed an expression of the relationship among experimental samples as detailed in the **Materials and Methods**. 87% of the early response through one month post primary immunization was composed

FIGURE 16.

IgG4 response in each of the non-human primates (NhP) after primary and secondary immunization with the protein tetanus toxoid. Development of the relative antibody levels as measured in ELISA Units over time. The lines represent the response development in M79 □, M99 +, P61 ◇, P69 △, R25 ✕ , and ▽ portrays the average response for all five monkeys. The points represent the mean value of triplicate determinations at each time interval.
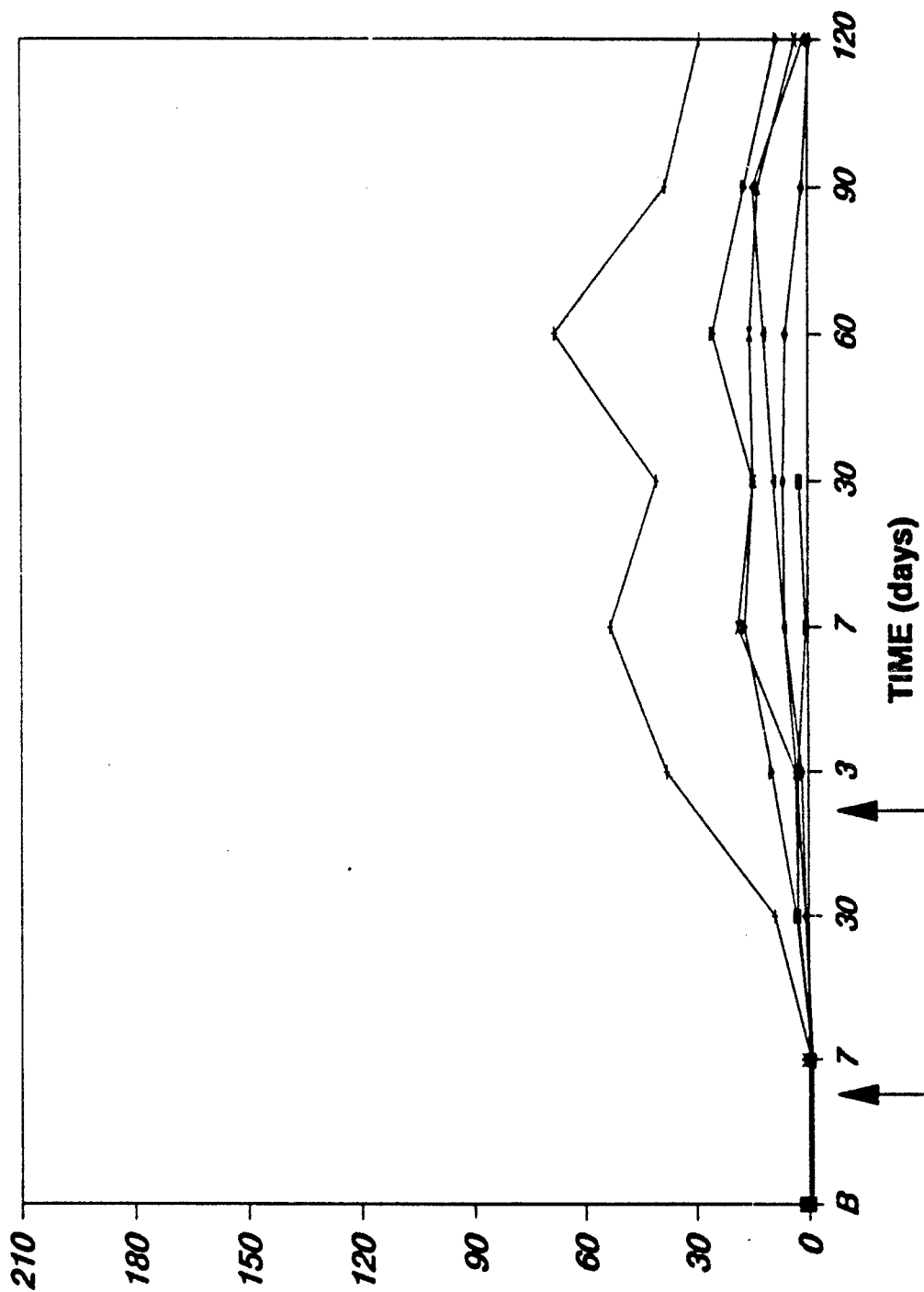
IgG4 RESPONSE IN NhP

TABLE 6

IgG4 Response in the Nonhuman Primate to Immunization
with Tetanus Toxoid

		PRIMARY		SECONDARY					
NhP	BASE	7	30	3	7	30	60	90	120
M79	0.26[a]	0.14	3.60	3.45	1.38	3.19	NT	NT	NT
M99	0.37	0.27	9.46	36.42	50.62	38.97	64.92	36.76	27.96
P61	0.13	0.17	1.62	2.52	6.71	7.14	6.65	2.49	0.42
P69	0.03	0.03	1.82	4.00	6.84	9.47	12.05	14.64	2.03
R25	0.09	0.06	3046	4.11	18.38	14.58	15.49	13.37	4.14
MEAN	0.17	0.13	3.99	10.10	16.79	14.67	24.78	16.82	8.64
SD	0.12	0.08	2.85	13.17	17.80	12.70	23.39	12.45	11.23

[a] denotes mean IgG antibody level expressed as ELISA Units (EU).
NT denotes not tested due to death of the animal unrelated to this study.

of IgG1 (62%) and IgG3 (25%)(**Figures 17 & 18 and Table 7**) During this time period, IgG4 and IgG2 participated minimally in the total IgG response (6.5% and 6%, respectively).

At 1 week to one month post secondary immunization, the response was still composed primarily of IgG1 and IgG3 (61% and 21% respectively). While IgG4 and IgG2 continued in lower proportions (9.5% and 7.4% respectively), the IgG4 did exhibit a somewhat selective change after the secondary immunization. Proportions at peak response in different monkeys varied between 40-65%, 16-28%, 7-24%, and 4-24% for IgG1, igG3, IgG4, and IgG2, respectively (**Figures 19-23**). Interestingly, at 2 or more months into the secondary response, IgG4 and IgG2 levels stabilized or increased slightly, while IgG1 and IgG3 levels were declining.

D. Development of IgG Avidity to Tetanus Toxoid in NhP

Studies concerning the development of antibody avidity with maturation of the immune response have described increasing avidity with time post-immunization. In general, two explanations have been proposed to explain this phenomenon. First, as the level of antigen available decreases during the immune response, those B cells producing high avidity (affinity) antibody compete more efficiently for antigen and continue to expand. The net result is an increased population avidity of the antibody. The second concept invokes the apparent ability of anti-idiotypes to regulate the immune response. In this regard, a decreased avidity of a particular antibody clonotype could be associated with greater serum concentration of free antibody (i.e., not effectively bound to an antigen). This would, in turn, elicit a greater anti-idiotype antibody response against these circulating low avidity clonotypes, leading to a down regulation of synthesis and thereby selecting for clonotypes with higher affinity for the antigen. However, no information is available which details the development of the binding strength of antibodies in nonhuman primates. Since both the antibody level and avidity are related to the effectiveness of the antibody, we designed studies to examine this parameter in the nonhuman primate in response to TT immunization.

FIGURE 17.

Development of the total IgG, IgG1, IgG2, IgG3, and IgG4 response in non-human primates after immunization with tetanus toxoid. The bars represent the mean peak level for each of the 5 NhP's at baseline, post-primary immunization, and post-secondary immunization.
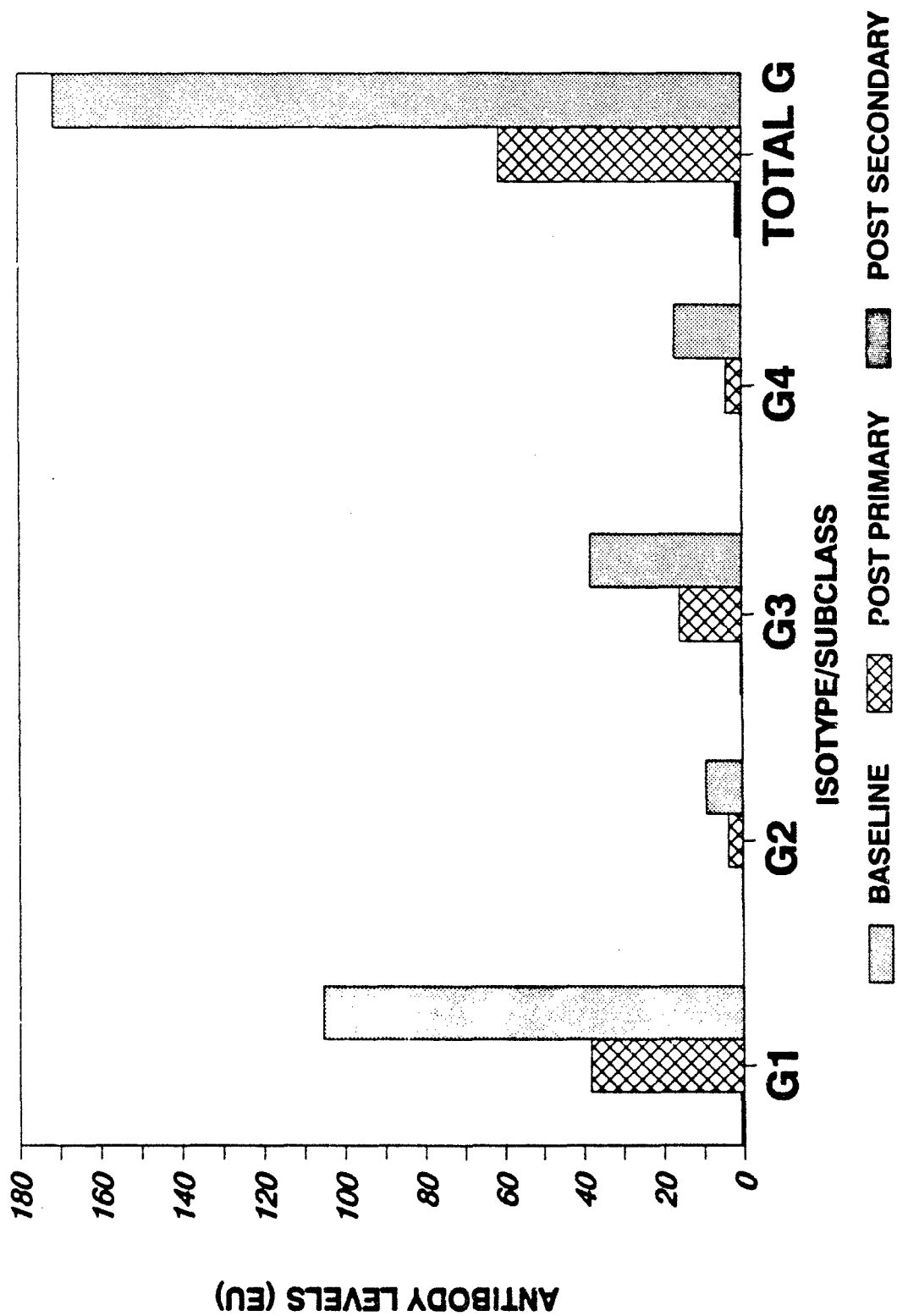
DEVELOPMENT OF IgG RESPONSE

ANTIBODY LEVELS (EU)

ISOTYPE/SUBCLASS

BASELINE POST PRIMARY POST SECONDARY

G1 G2 G3 G4 TOTAL G

FIGURE 18.

IgG subclass representation in the total IgG antibody response to tetanus toxoid. The points denote the mean level of IgG $\times$, IgG1 $\square$, IgG2 $+$, IgG3 $\diamond$, IgG4 $\triangle$ in the group of five monkeys. The data reflect an expression of each subclass proportionally related to the total IgG response.

SUBCLASS PROPORTION OF TOTAL RESPONSE

ANTIBODY LEVELS (EU)

TIME (days)

TABLE 7
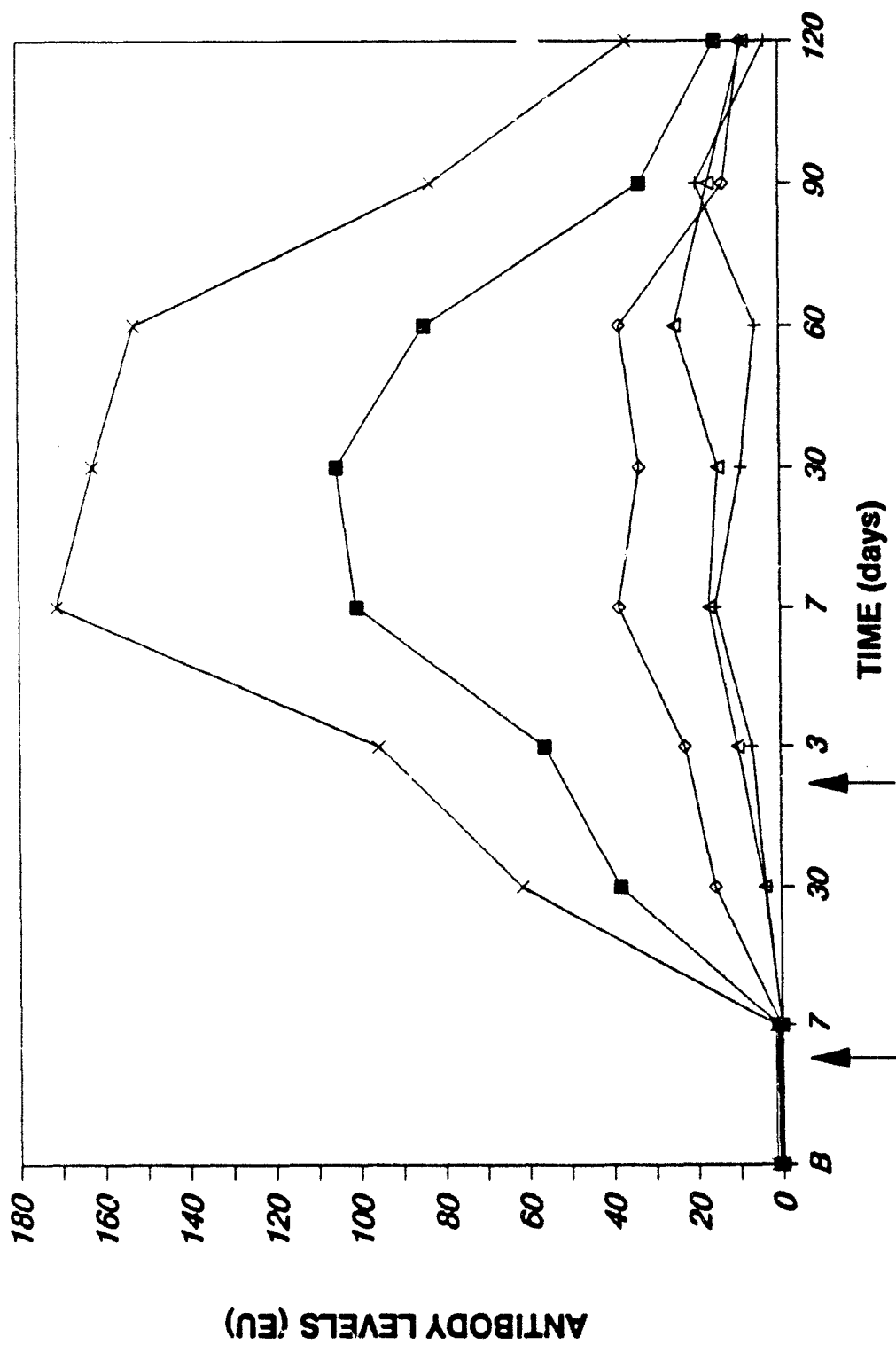
Subclass Proportion of Total IgG Response in the Nonhuman Primate to Immunization with Tetanus Toxoid

Isotype	BASE	PRIMARY		SECONDARY					
		7	30	3	7	30	60	90	120
IgG	1.55[a]	1.56	61.41	95.50	171.19	162.65	152.52	82.72	36.33
IgG1	0.796 (51%)	0.90 (58%)	38.09 (62%)	56.04 (59%)	100.74 (59%)	105.41 (65%)	84.36 (55%)	33.05 (40%)	15.14 (42%)
IgG2	0.15 (10%)	0.15 (10%)	3.74 (6%)	6.72 (7%)	15.39 (9%)	9.28 (6%)	5.63 (4%)	19.58 (24%)	3.41 (9%)
IgG3	0.44 (28%)	0.37 (24%)	15.60 (25%)	22.63 (24%)	38.27 (22%)	33.29 (20%)	37.93 (25%)	13.27 (16%)	9.14 (25%)
IgG4	0.17 (11%)	0.13 (9%)	3.99 (7%)	10.10 (11%)	16.79 (10%)	14.67 (9%)	24.78 (16%)	16.82 (20%)	8.64 (24%)

[a] denotes mean IgG antibody level expressed as ELISA Units (EU) related as a proportion of the total IgG response. Values in parentheses represents the % of the total response comprised by each subclass.

NT denotes not tested due to death of the animal unrelated to this study.

IGURE 19.

₃G subclass representation in the total IgG antibody response to tetanus toxoid in NhP
Λ79. The points denote the mean level of IgG ✕ , IgG1 □, IgG2 ┿, IgG3 ◇, IgG4 △.
he data reflect an expression of each subclass proportionally related to the total IgG
esponse.

IgG and SUBCLASS RESPONSES IN NhP (M79)

FIGURE 20.

gG subclass represer.tation in the total IgG antibody response to tetanus toxoid in NhP M99. The points denote the mean level of IgG ✕ , IgG1 ▢, IgG2 ✚, IgG3 ◇, IgG4 △. The data reflect an expression of each subclass proportionally related to the total IgG response.

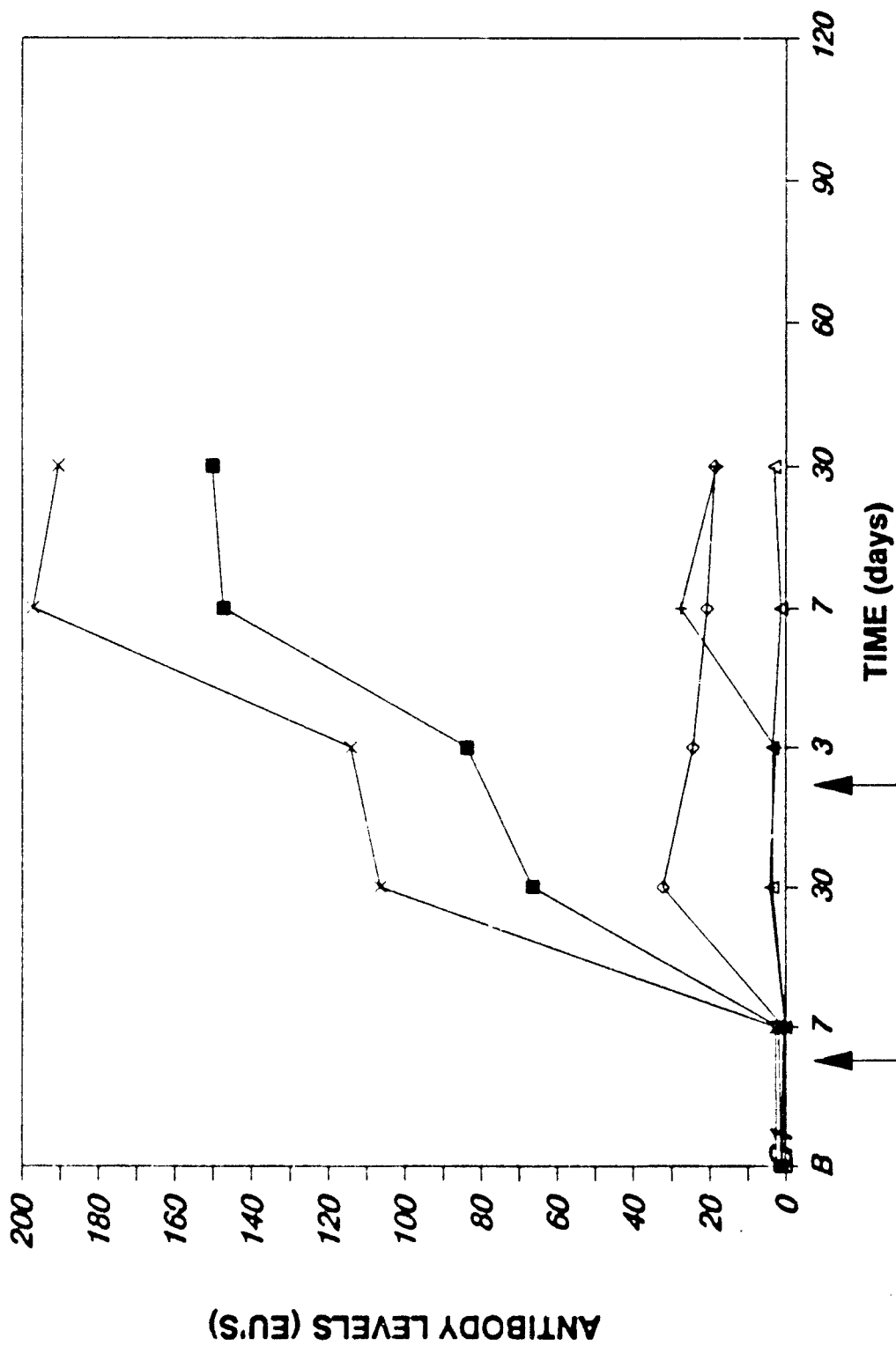IgG and SUBCLASS RESPONSES IN NhP (M99)

FIGURE 21.

IgG subclass representation in the total IgG antibody response to tetanus toxoid in NhP
P61. The points denote the mean level of IgG $\times$, igC1 $\square$, IgG2 $+$, IgG3 $\Diamond$, IgG4 $\triangle$. The
data reflect an expression of each subclass proportionally related to the total IgG response.
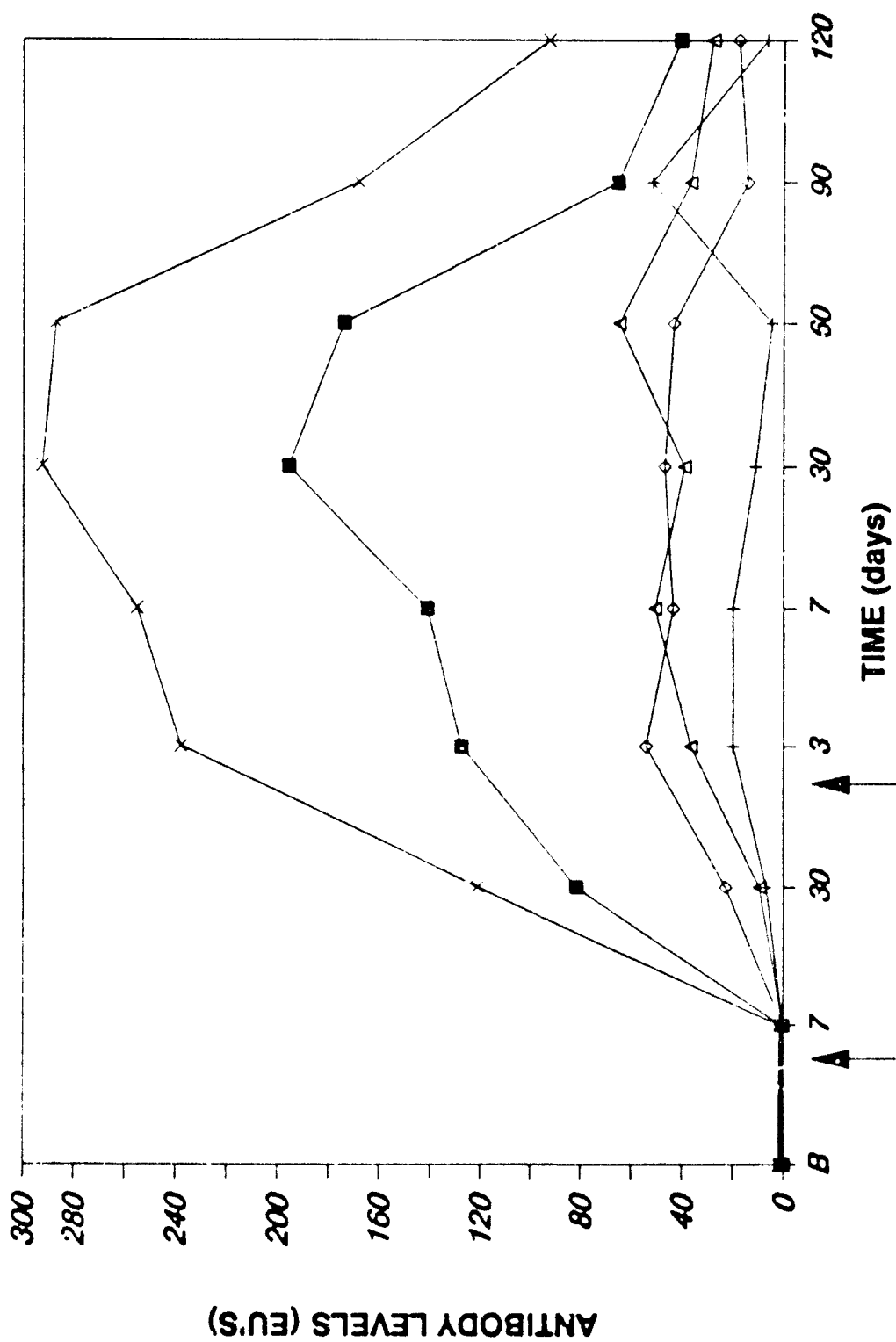
IgG and SUBCLASS RESPONSES IN NhP (P61)

ANTIBODY LEVELS (EU's)

TIME (days)

FIGURE 22.

IgG subclass representation in the total IgG antibody response to tetanus toxoid in NhP

P69. The points denote the mean level of IgG $\times$, IgG1 $\square$, IgG2 $+$, IgG3 $\Diamond$ IgG4 $\triangle$. The

data reflect an expression of each subclass proportionally related to the total IgG response.

IgG and SUBCLASS RESPONSES IN NhP (P69)
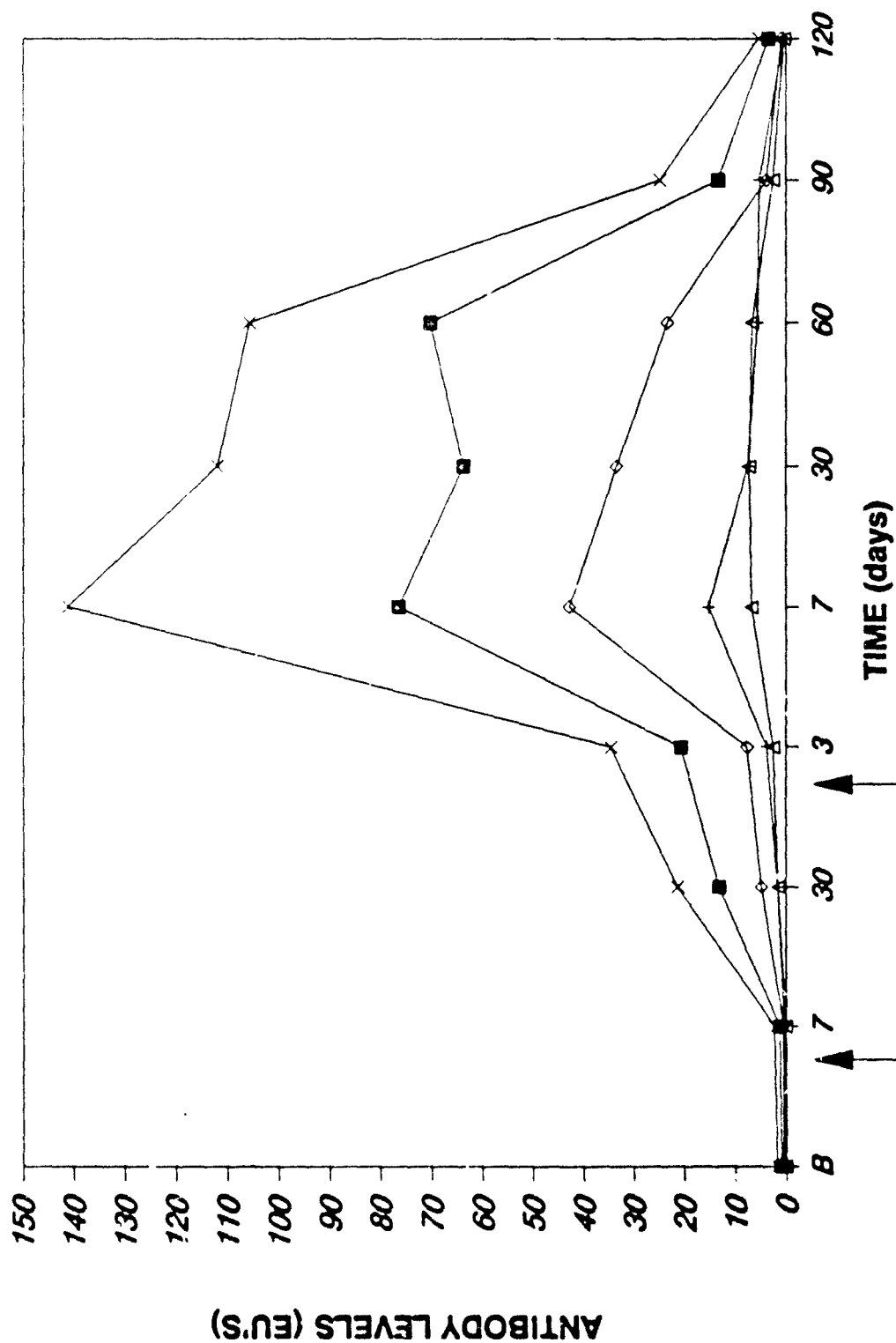

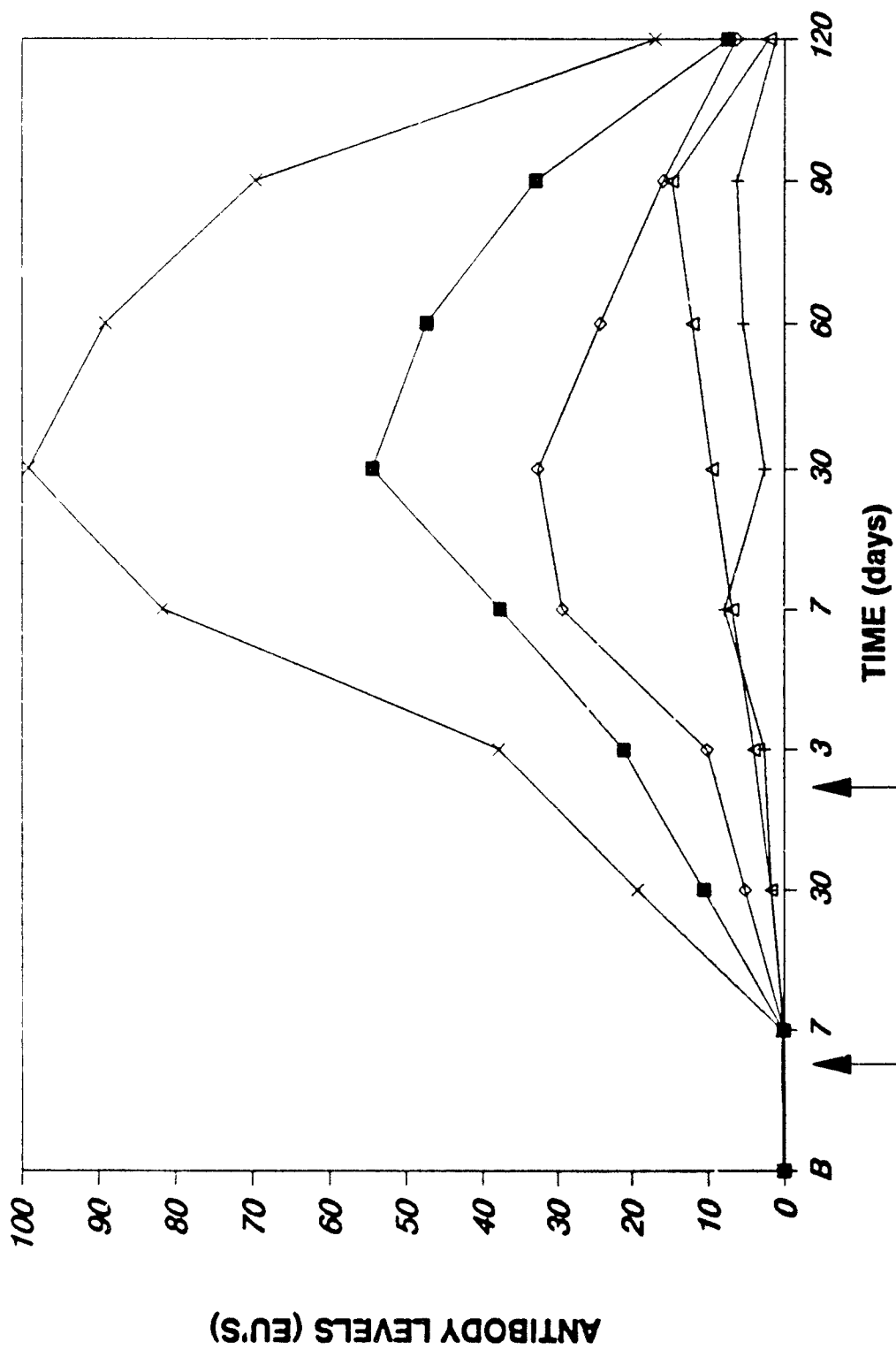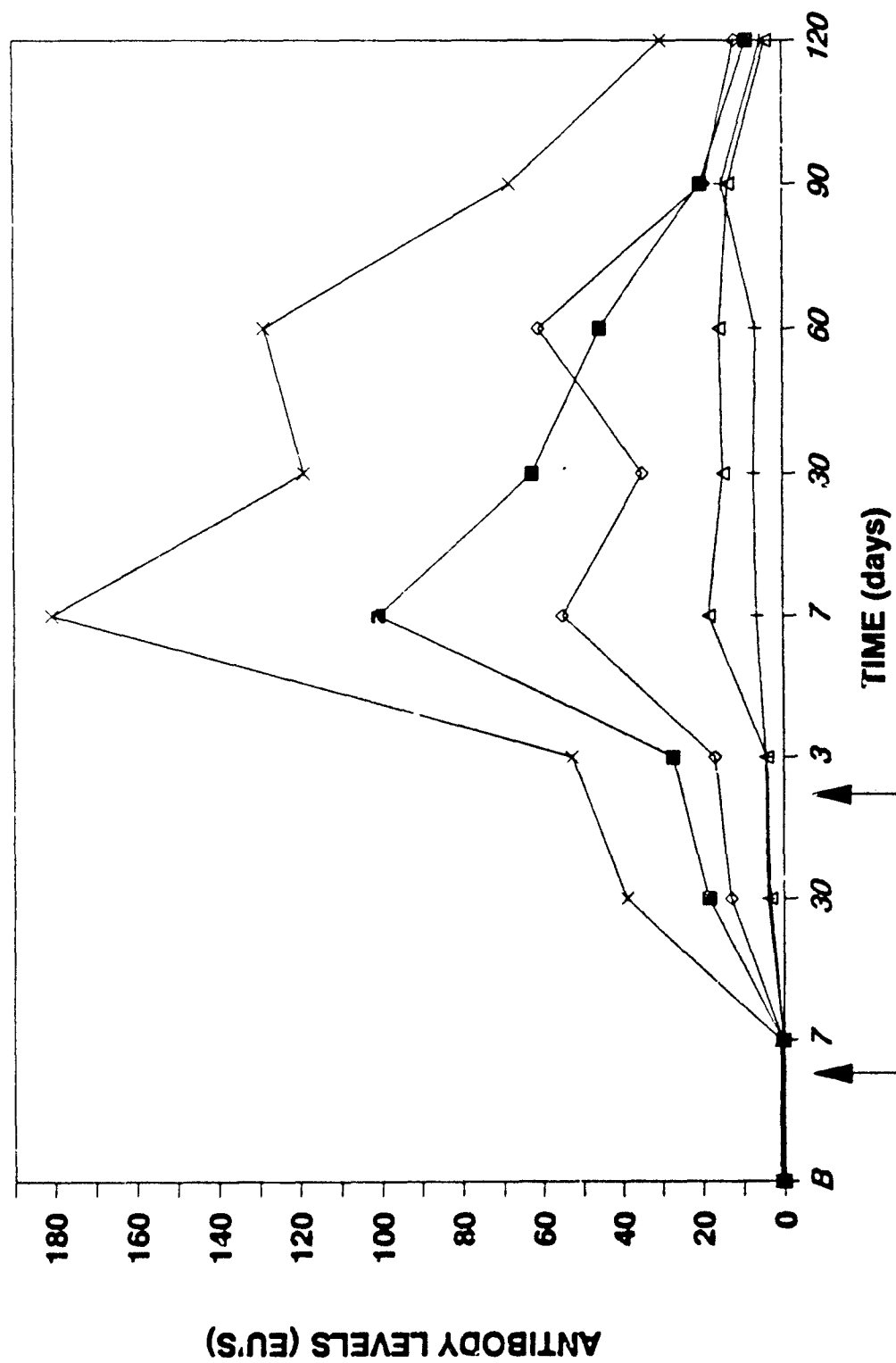
ANTIBODY LEVELS (EU'S)

TIME (days)

FIGURE 23.

IgG subclass representation in the total IgG antibody response to tetanus toxoid in NhP R25. The points denote the mean level of IgG $\times$, IgG1 $\square$, IgG2 $+$, IgG3 $\diamond$, IgG4 $\triangle$. The data reflect an expression of each subclass proportionally related to the total IgG response.

IgG and SUBCLASS RESPONSES IN NhP (R25)

Experiments were performed to examine the ability of the chaotropic ions to elute antibody from the TT antigen. The results in **Figures 24-28** depict the elutiony profiles for each of the immunized profiles. Included in each figure is the profile of antibody binding strength from baseline, primary and secondary immunization studies. The results indicate that as the serum antibody response progressed through the primary and secondary immunizations, the inhibiting portion of each curve (i.e. slope decreasing from 0 molar eluant) was noted to move further towards the higher concentrations of NH_4SCN. These results indicate a stronger binding between the antigen and the antibody population contained in the serum.

Baseline IgG avidity indices (AI) to TT in the various NhP's measured from 0.81 to 1.05 (**Figure 29 and Table 8**) Primary immunization increased the AI to 1.7 $\pm$ 0.47 at 1 month. Maximum avidity (AI ~2.6) was achieved 3 months post secondary immunization in all animals which was significantly greater than baseline ($p < 0.02$). Although peak avidity was not coincident with peak antibody levels, the overall increase in both was positively correlated ($p < 0.002$). The increase in avidity between the primary and secondary immunizations was statistically significant ($p < 0.02$).

FIGURE 24.

Elution profile of anti-TT IgG antibody in NhP M79 at baseline □, after primary immunization: 30d + ,; and after secondary immunization: 7d ◇, 30d △, 60d ✕, and 90d ▽. The points represent duplicate determinations of the effect of antibody binding to the TT antigen at each molar concentration of the eluant.

ELUTION CURVE CHANGES IN M79
WITH TIME POST IMMUNIZATION

ELUANT CONCENTRATION

LOG$_{10}$ % REACTIVITY of CONTROL

FIGURE 25.

Elution profile of anti-TT IgG antibody ir VhP M99 at baseline □ , after primary immunization: 30d + „; and after secondary immunization: 7d ◇, 30d △, 60d ✕, and 90d ▽. The points represent duplicate determinations of the effect of antibody binding to the TT antigen at each molar concentration of the eluant.

ELUTION CURVE CHANGES IN M99
WITH TIME POST IMMUNIZATION

LOG$_{10}$ % REACTIVITY of CONTROL

ELUANT CONCENTRATION

FIGURE 26.

Elution profile of anti-TT IgG antibody in NhP P61 at baseline □ , after primary immunization: 30d + ; and after secondary immunization: 7d ◇ , 30d △ , 60d ✕ , and 90d ▽. The points represent duplicate determinations of the effect of antibody binding to the TT antigen at each molar concentration of the eluant.

ELUTION CURVE CHANGES IN P61
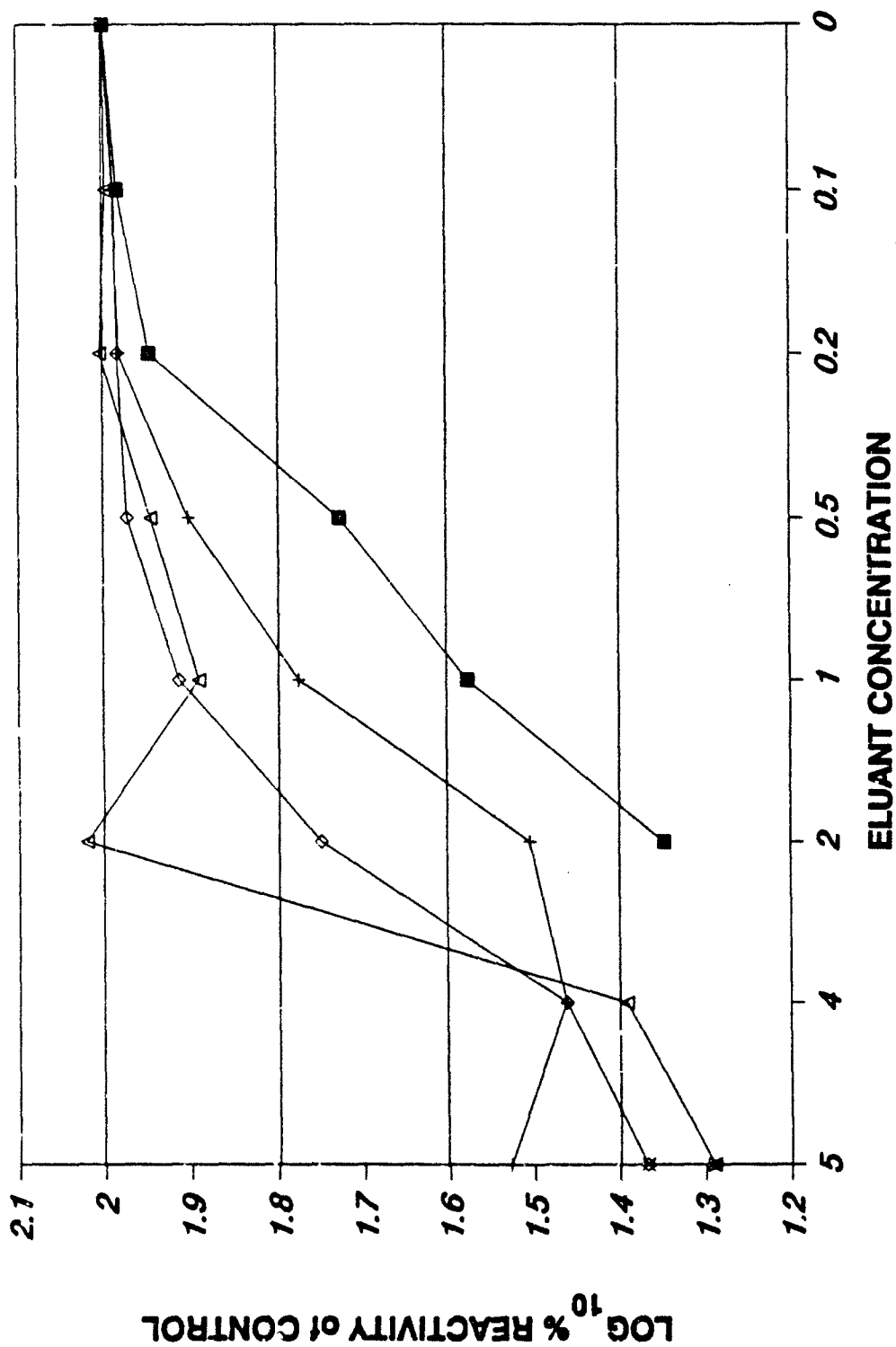WITH TIME POST IMMUNIZATION
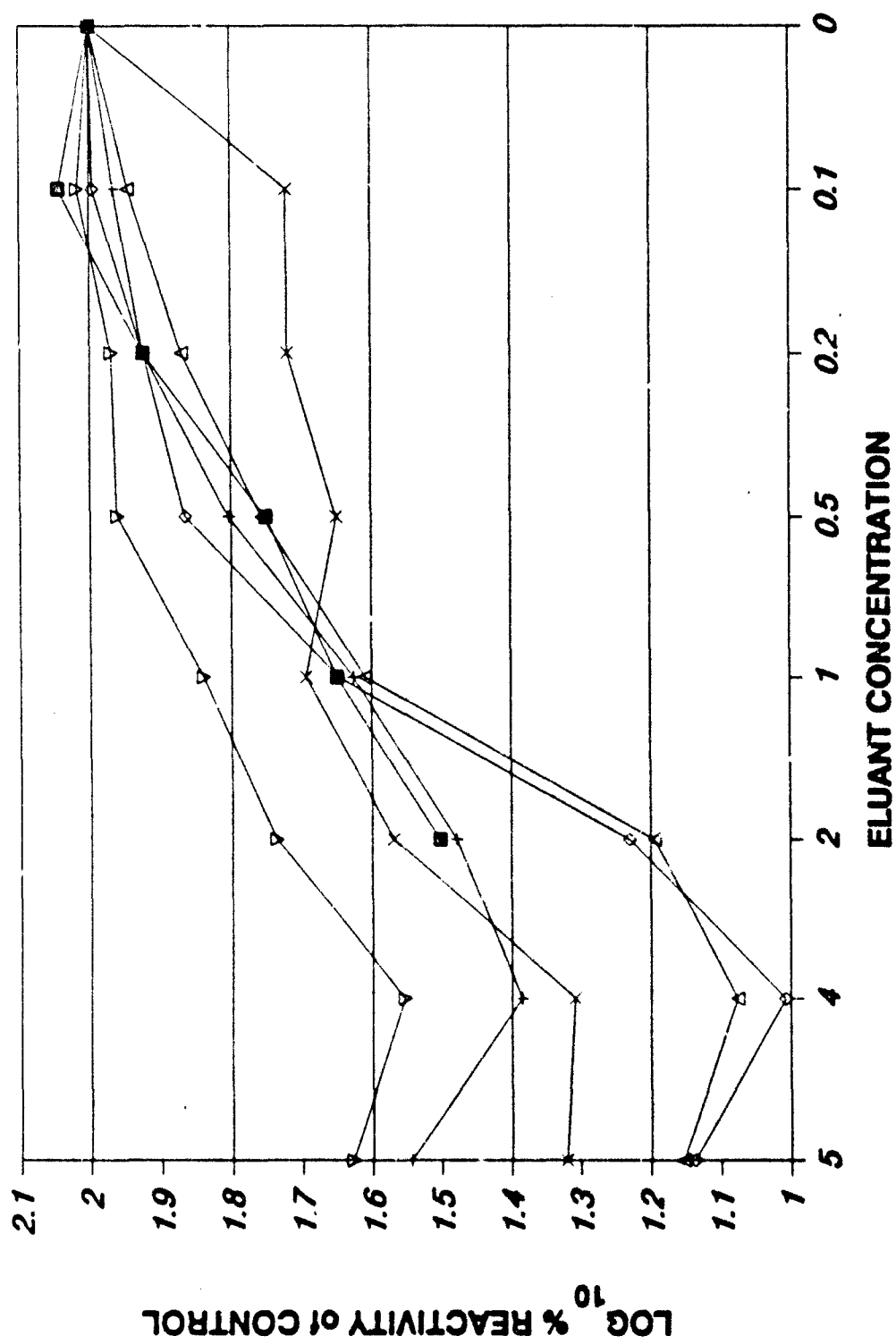
FIGURE 27.

Elution profile of anti-TT IgG antibody in NhP P69 at baseline □ , after primary immunization: 30d + ,; and after secondary immunization: 7d ◇, 30d △, 60d ✕, and 90d ▽. The prints represent duplicate determinations of the effect of antibody binding to the TT antigen at each molar concentration of the eluant.

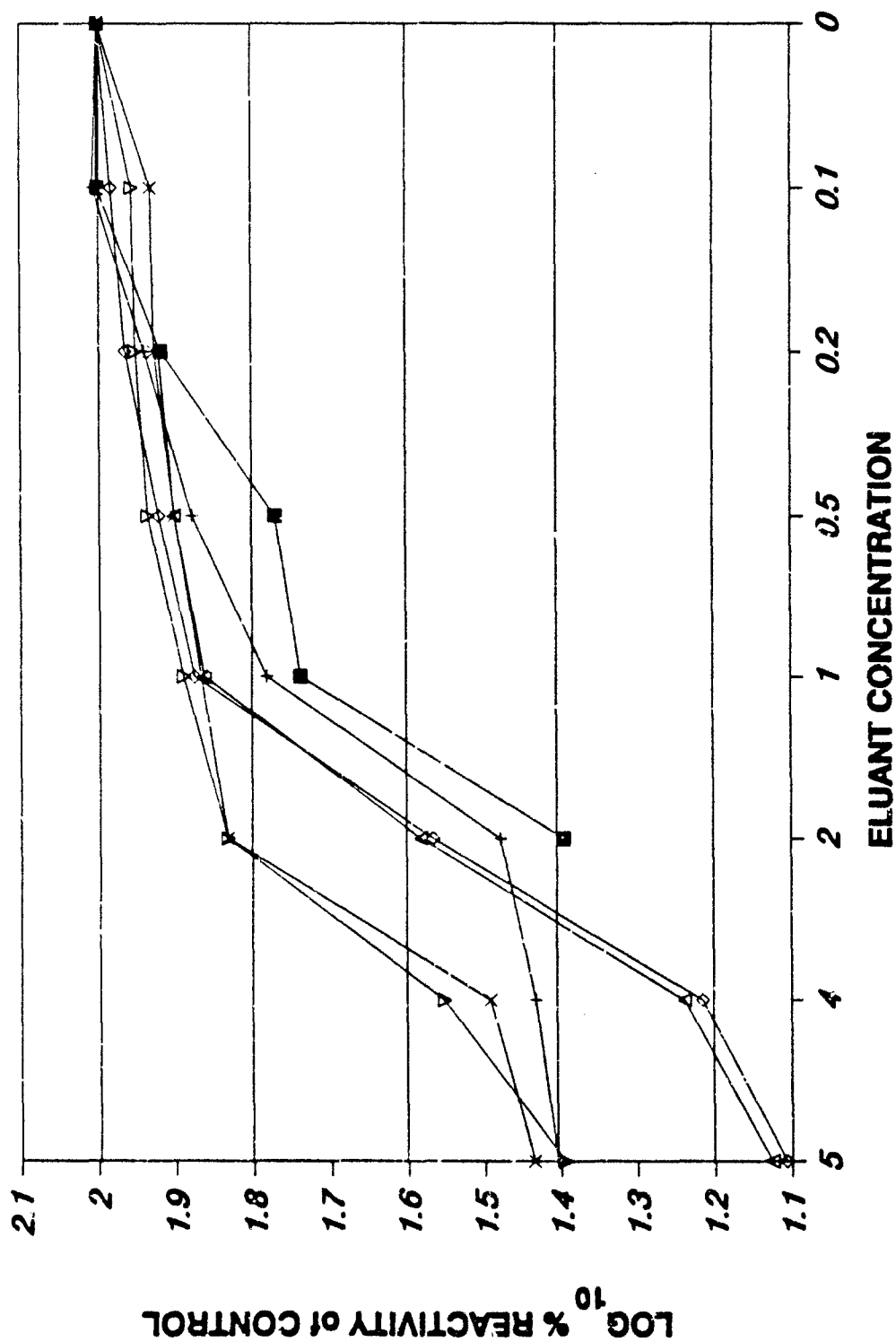ELUTION CURVE CHANGES IN P69 WITH TIME POST IMMUNIZATION
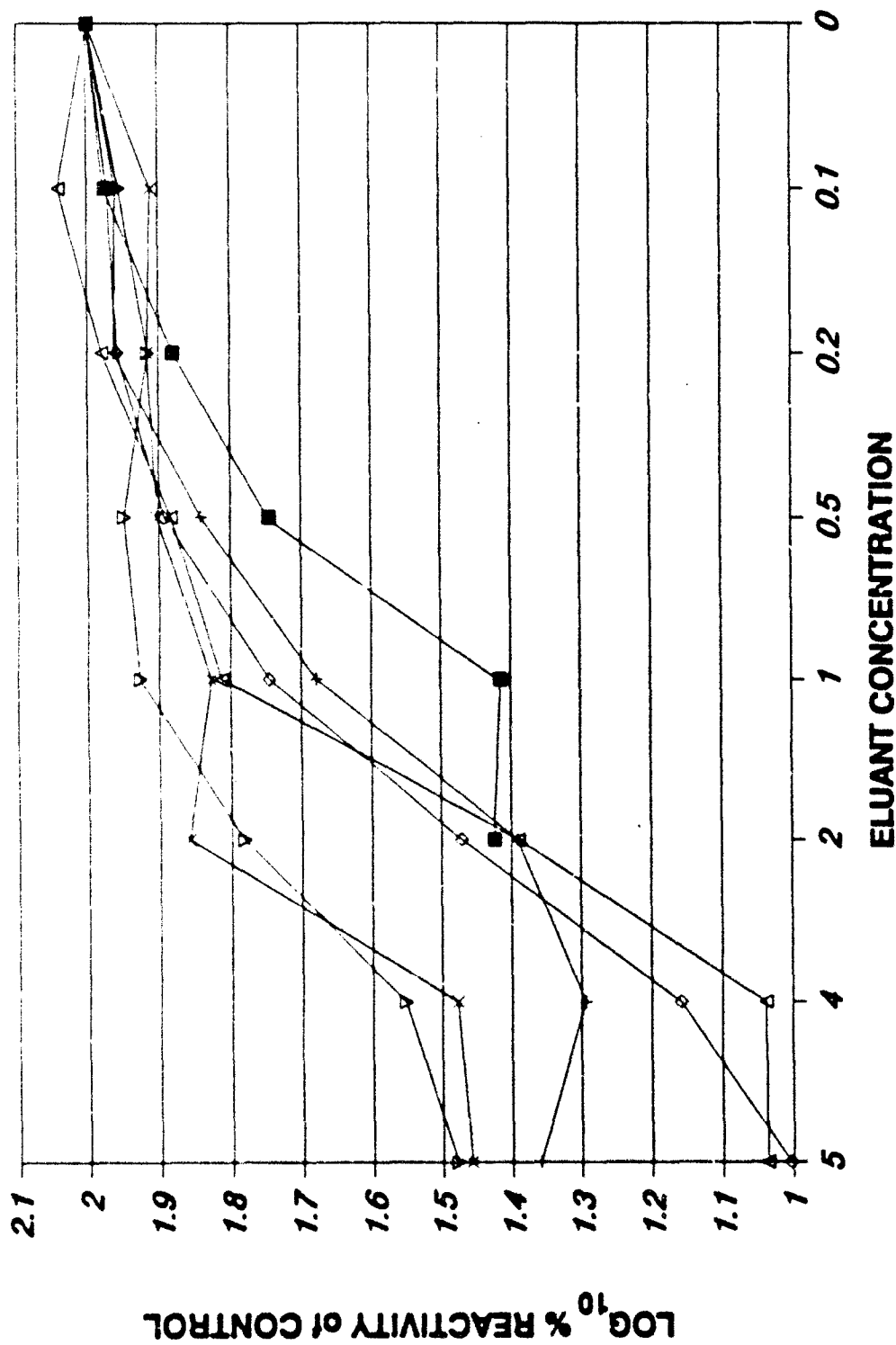
FIGURE 28.

Elution profile of anti-TT IgG antibody in NhP R25 at baseline □, after primary immuniza-
tion: 30d + ,; and after secondary immunization: 7d ◇, 30d △, 60d ✕, and 90d ▽. The
points represent duplicate determinations of the effect of antibody binding to the TT
antigen at each molar concentration of the eluant.

ELUTION CURVE CHANGES IN R25 WITH TIME POST IMMUNIZATION

LOG_{10} % REACTIVITY of CONTROL

ELUANT CONCENTRATION

FIGURE 29.

Development of antibody avidity over time in the non-human primate in response to immunization with the protein tetanus toxoid. Each line of interconnected symbols represents the avidity index at specified time points in M79 □, M99 +, P61 ◇, P69 △, R25 ✕, and ▽ portrays average avidity index for all monkeys.

AVIDITY DEVELOPMENT WITH TIME

AVIDITY INDEX

TIME (days)
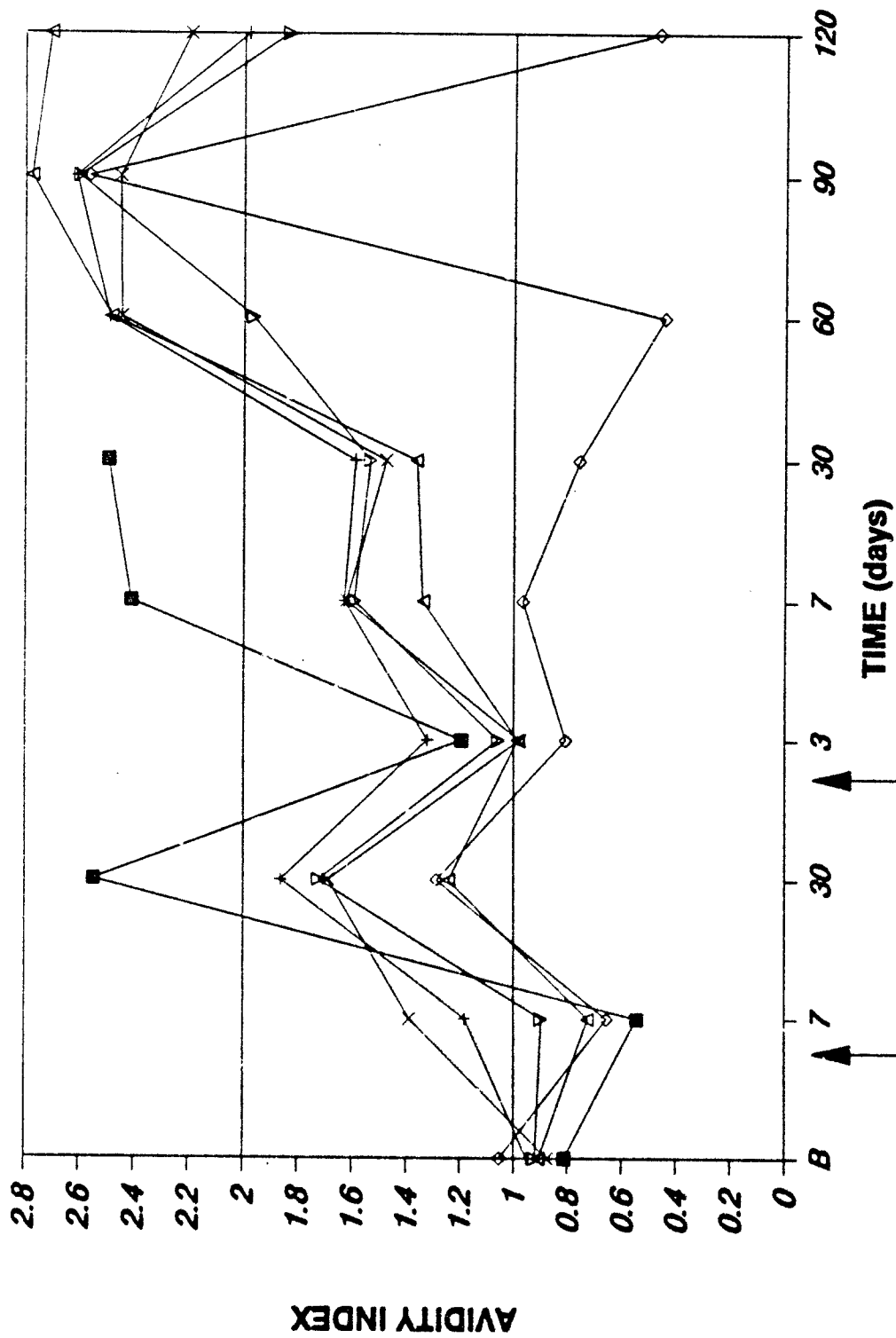
TABLE 8

Avidity Maturation in the IgG Response to Tetanus Toxoid
in the Nonhuman Primate

NhP	BASE	PRIMARY		SECONDARY					
		7	30	3	7	30	60	90	120
M79	0.81[a]	0.54	2.55	1.19	2.41	2.5	NT	NT	NT
M99	0.95	1.18	1.86	1.32	1.63	1.59	2.49	2.61	1.98
P61	1.05	0.66	1.29	0.81	0.97	0.76	0.44	2.56	0.47
P69	0.91	0.72	1.24	0.98	1.34	1.36	2.48	2.77	2.7
R25	0.88	1.39	1.69	0.98	1.62	1.48	2.45	2.45	2.19
MEAN	0.92	0.89	1.72	1.06	1.59	1.54	1.97	2.59	1.83
SD	0.08	0.33	0.47	0.18	0.47	0.56	0.88	0.12	0.83

[a] denotes mean Avidity Index (AI) of IgG antibody.
NT denotes not tested due to death of the animal unrelated to this study.

IV. DISCUSSION AND SUMMARY

In this study an ELISA was developed for characterization of the IgG immune response in the nonhuman primate following immunization with the prototype protein toxin, tetanus toxoid (TT). Seven nonhuman primates were administered a primary and secondary immunization with TT, two of which were used to develop reference serum. Serum samples were collected from the other five monkeys through one month post-primary immunization and up to four months post-secondary immunization. The validity of using mouse monoclonal antibodies, which had been developed for detection of human IgG subclasses, to detect monkey IgG subclasses was confirmed by ELISA inhibition analysis. Serum samples were then evaluated by ELISA for IgG and IgG subclass antibody levels and development of IgG antibody avidity post-immunization with TT.

After initial determination of the appropriate concentration of antigen (tetanus toxoid) for plate coating and serum sample dilutions, there remained sporadic OD signals from random wells suggesting nonspecific binding. Following experimentation with various blocking methods, appropriate concentrations of gelatin and non-fat powdered milk were selected to control this problem. Subclass ELISA's required greater concentrations of antigen coating and serum samples. This should not be unexpected since identification of total IgG by ELISA involves a signal from all subclasses, whereas identification of one of the four subclasses yields only a portion (3-79%) of the signal obtained in the total IgG response.

Total IgG response to the NhP to the protein TT was examined using affinity purified goat anti-human IgG polyclonal sera. Anti-human IgG serum has been used in the past for detection of monkey serum IgG with apparent satisfactory cross reactivity and specificity preserved (Monte-Wicher 1970). The present study further supported the use of anti-human IgG in NhP IgG detection studies by ELISA inhibition analyses. Briefly, the ability of goat anti-human IgG to identify, similarly, human anti-TT serum binding to tetanus toxoid coated microtiter plates versus NhP anti-TT binding to equivalently coated plates was evaluated. While direct comparison of antibody levels between human and NhP serum can not be supported by the results of this analysis, human and NhP anti-TT dilution curves were very similar.

The total IgG response post-primary immunization was significant at one month exhibiting a 40 fold increase in antibody levels over baseline. There was no increase in activity detected at one week post-primary immunization. These results indicated that these NhP were generally naive to this antigen. However, since no serum samples were collected between these two time points, it is unknown whether the primary response would have been detectable in this interval. In future studies attempting to characterize the response to protein antigenic challenge in this model, additional serum samples might be collected in the one week to one month interval to more clearly delineate the kinetics of the primary response.

A significant secondary response was also noted, the peak of which occurred at one week to one month post-secondary immunization, and demonstrated a 110-fold increase over baseline. Though total IgG response levels varied quantitatively, the pattern of response was similar for all animals with a peak in primary response at one month post-primary immunization, a peak in secondary response at one week to one month post-secondary immunization, and the response in all animals was in decided decline after two months post-secondary immunization. At termination of the study, antibody levels had not assumed a level similar to the baseline state. Therefore, it might be useful, in the future, to extend the period of serum collection to determine the interval for achieving steady-state levels.

Donnenberg (1984) collected serum from 7 healthy human subjects before and up to 180 days after secondary immunization with tetanus toxoid. Anti-tetanus IgG levels were determined by ELISA. Maximum IgG secondary response occurred between one week to one month post-secondary immunization which was the time point of maximum secondary response for the present study. In some individuals, IgG anti-tetanus toxoid activity was still elevated at 180 days post-immunization, while in others, activity returned to baseline levels. Future studies might include later time points for serum sampling to gather data on subjects with a lingering response as noted by Donnenberg. Remarkably, a significant decrease in circulating anti-tetanus IgG levels were noted on day one post-secondary immunization which in some cases continued through day seven. The reduction is attributed to anti-anti-tetanus antibodies (anti-idiotypes) which could complex with

the anti-tetanus immunoglobulins thus clearing them from circulation. A similar decrease in circulating IgG was not noticed in the present study. However, the rate of increase in anti-tetanus antibody levels, continuous with the primary response, was decreased at 3 days post-secondary immunization. Whether this was due to an ebb in the primary response, or a booster related phenomenon was not able to be determined. A decrease in avidity was seen at three days post-secondary immunization in the current study, and will be discussed later.

In another study, Farzad (1985) determined pre- and post-secondary IgG antibody levels in 3 human subjects up to six months after boosting with tetanus toxoid. By ELISA, a peak response was seen at seven to fourteen days post-immunization, which was still elevated at 6 months. No drop in levels was noted at the earliest time interval (five days) post-immunization in any of these patients.

Investigations into the validity of using mouse anti-human IgG subclass monoclonals to detect the NhP IgG subclasses revealed a substantial amount of cross-reactivity between the human and monkey molecules (45-85%) with conserved epitopes for detection of the analogous subclasses in each species. This was accomplished by three groups of ELISA inhibition analyses in which one phase (human IgG subclass myeloma protein, human anti-TT reactive serum, or mouse anti-TT reactive serum) was initially bound to the microtiter plate either directly or indirectly. The second phase was then added, consisting of the detecting monoclonal antibody (human IgG subclass directed) and an inhibiting co-incubant (either human anti-TT non- reactive serum, or monkey anti-TT non-reactive serum, or human IgG subclass myeloma protein) which was applied to the plate prior to completion of developing. The amount of co-incubant related inhibition of monoclonal-phase 1 binding was related to similarities between monkey and human IgG subclass antigenic structure.

The plate-bound phase in the first analyses consisted alternately of each of the particular IgG subclass myeloma proteins. Binding of the human IgG subclass-directed monoclonal antibody to the first phase was measured with and without human or monkey serum co-incubation prior to completion of ELISA development. The results of these analyses demonstrated significant inhibition of monoclonal binding to human mye-

loma proteins when either monkey or human serum was used as the inhibitor. However, the human serum was always more inhibiting than monkey serum. This was probably related to the greater specificity of the monoclonal for human IgG subclasses. The fact that monkey serum was inhibitory does suggest a significant degree of specificity of the monoclonal for monkey IgG subclasses also. The monkey IgG serum was about 45-85% as inhibitory as human serum for the various subclasses in these analyses.

In the second group of inhibition analyses, the plate bound phase was monkey or human Anti-TT (which was bound to the plate by previous TT coating). The subclass specific monoclonal in these analyses was co-incubated with the homologous IgG subclass human myeloma protein or was incubated alone prior to completion of ELISA development. The inhibition of monoclonal binding to monkey anti-TT serum by the human IgG subclass myeloma protein was similar to inhibition of the monoclonal to human anti-TT when concentrations of detectable anti-TT were equivalent. This further suggests antigenic similarity between monkey and human IgG subclass molecules.

The third group of inhibition analyses also began with plate-bound monkey or human anti-TT. However, in this case, human or monkey anti- TT-negative sera was used as the inhibitor of monoclonal-phase 1 binding. In this inhibition study, both monkey and human anti-TT negative serum demonstrated significant monoclonal-phase 1 inhibition. In every case, the NhP serum was less inhibitory than human serum indicating less than total cross reactivity. However, significant inhibition of monoclonal-human IgG subclass binding by monkey non- reactive serum is indicative of antigenic cross reactivity between monkey and human IgG subclass molecules.

There is no direct method for determining equal levels of subclasses between the two species sera. However, it could be suggested from this data that IgG3 and IgG4 inhibiting ability were more similar due to greater cross-reactivity in antigen epitopes between the species. The results of these analyses suggest significant homology between human and NhP IgG subclass antibodies with the greatest epitopic similarity in IgG3 and IgG4 subclasses and somewhat less with IgG1 and IgG2.

Epitopic similarities between antibody markers have been reported across closely related vertebrates, and especially within a family of species (Mota 1986). Monte-Wicher

(1970) studied the antigenic relationship between monkey and human IgM, IgG, and IgA using monospecific polyclonal antisera produced in the rabbit against these Ig isotypes in the monkey and another against human Ig isotypes. Each antiserum was cross reacted with homolgous and heterologous sera, and the amount of antigenic similarity determined by double diffusion gel precipitation. Cross-reaction was near equivalent between IgM isotypes but varied between the other two classes. IgA was the least antigenically similar between the two species. Anti-human IgG cross-reacted better with monkey IgG (57-68%) than did anti-monkey IgG with human isotypes 56-69%. Comparison of cross-reactivity by single radial diffusion suggested that monkey IgG contained additional antigenic binding sites over human IgG. Although greater epitopic homology between anti-human IgG with monkey IgG would be more ideal, Martin (1982) reported variability of IgG Fab constant regions even within a single species of monkey. Additional support for antigenic similarities between human and monkey immunoglobulins is the discovery of shared Ig allotypic markers in human and NhP Ig constant regions, especially on IgG subclass heavy chains (van Loghem 1986). These allotypic markers are genetically determined and have been related to variation in antibody response to certain antigens.

IgG subclass levels were evaluated in the present study by ELISA using mouse anti-human IgG subclass monoclonals. The IgG subclass response to proteins has not been characterized in the NhP previously, and theref... primate data does not exist with which to compare data in this study. However, extensive investigation of the human response to tetanus toxoid allows comparison of the NhP response to that of man. Future investigations of the NhP IgG subclass response are needed to corroborate the data from this study.

In the present investigation, all subclasses showed some response to the protein toxin. This is not in disagreement with other studies although some report an absence of significant IgG2 response (Seppala 1984, Bird 1984). These studies looked only at levels post-secondary immunization without comparison to baseline levels existing before primary immunization. Some IgG2 was always present but was not considered to be a significant 'participant' in the response. van der Geissen did evaluate pre-immunization antibody levels, and reported significant response levels in all subclasses relative to

baseline though very low levels of IgG2 presented.

In the present investigation, IgG1 was the dominant IgG subclass, comprising 62% of the response in the NhPs. IgG3 was also a major participant contributing 25% of the response. IgG2 and IgG4 played only minor roles (6% & 6.5%, respectively). The absolute response levels in IgG2 and IgG4 subclasses were very low and the reported significant increase inlevels post-primary and secondary immunization is only in relation to their exceptionally low baseline levels and, later, their continued activity in the face of declining IgG1 and IgG3.

Seppala (1984) investigated the IgG, and IgG subclass response in humans to booster immunization with tetanus toxoid by radioimmunoassay and found IgG1 (91%) and IgG4 (6.9%) to be the only significant reactors. Levels of IgG2 and IgG3 were so low as to be considered insignificant. Pre-immunization levels were not recorded.

van der Giessen (1976), measured anti-tetanus IgG subclass levels before and after secondary immunization in six healthy human subjects up to three months. Using a quantitative immunofluorescence method known as defined antigen substrate spheres, the subclass proportional response by relative fluorescence intensity. He described significant antibody activity in all four IgG subclasses, though IgG1 was far and away the highest responding participant (51%). IgG4 levels were second highest (20%) followed by IgG3 (16%) and IgG2 (13%). Maximum response was reported at fourteen to twenty-eight days post-immunization for all subclasses. This is at variance with our findings in the monkeys which showed a later peak in IgG2 and IgG4 levels at two and three months, respectively, post-immunization. IgG4 and IgG2 levels were reported by van der Giessen to decline very slowly after secondary response just as in this study.

The importance of avidity in functional adequacy of the response is exemplified in a report by Passen (1983) in which a patient exhibited clinical tetanus despite "protective" levels of toxin-neutralizing antibody. An ample response in levels of antibody does not necessarily confer functional adequacy. The response must fulfill three criteria in order to confront the antigenic challenge successfully: 1) adequate antibody levels, 2) antibodies with specificity for the particular antigen involved, and 3) sufficient avidity to result in binding of the antigen (Steward & Steensgaard 1983). The present study defined the lev-

els as well as the avidity of antibodies produced in response to immunization with a prototype protein antigen. Specificity of response to a given protein antigen might best be accomplished relative to the environment in question. For instance, specificity of antiserum to the leukotoxin of *A. actinomycetemcomitans* might best be evaluated relative to protein factors in the periodontal environment, i.e., local bacterial and host proteins.

IgG avidity was determined by a modified ELISA using a chaotropic ion (NH_4SCN) to disrupt antigen-antibody interactions in proportion to the strength of the antibody-antigen binding. An avidity index (AI) was determined for each experimental sample and defined as that molar concentration of the eluant which was required to reduce the OD of antibody binding by 50%. Antibody avidity increased with time post-immunization for all NhPs. The avidity peaked at one month post-primary immunization and again at two to three months post-secondary immunization. Interestingly, antibody avidity declined early after secondary immunization in most of the NhPs before climbing again to maximum levels. This effect has been attributed to the formation of antigen-antibody complexes which are rapidly cleared through the kidney. These complexes involve greater proportions of high avidity antibody because of their preferential affinity for the antigen. The result is a decrease in overall antiserum avidity (Humphrey 1963). As mentioned previously, Donnenberg (1984) noticed a drop in antibody levels similarly at up to one week after boosting. He calculated that the amount of antibody population that could be cleared as a consequence of the small immunizing dose used would not be sufficient to account for the drop in antibody levels encountered in his study. He therefore attributed the decrease in antibody levels to the clearing of tetanus specific antibody by anti-idiotypes.

Although antibody levels and avidity increased post-primary and secondary immunization, peak levels and avidity were not coincident. During the primary response, antibody levels peaked at one month post-immunization and continued to increase after secondary immunization. Although avidity also peaked at one month post-primary immunization, a rapid decline was noted one week after boosting to near baseline levels. Furthermore, secondary response antibody levels peaked at one week to one month after boosting, whereas peak in avidity did not occur until the second to third month. When the changes in antibody levels and avidity were subjected to Spearman Rank Correlation

analysis, the two were correlated (p < 0.05) but independent of time. Independence of antibody level and avidity development has been reported by others (Pullen 1986, Brown 1984, Hedman 1988, Inouye 1984). Furthermore, avidity may remain low throughout a fairly high titer response and vice versa (Lew 1988). The weight of evidence would indicate that avidity is not bound to absolute level of antibody response.

In conclusion, immunization of nonhuman primates with the protein antigen, tetanus toxoid resulted in a significant IgG response over baseline post-primary and secondary immunization, which declined three months post-secondary immunization. Inhibition ELISAs in this study supported the use of mouse anti-human IgG subclass specific monoclonals to detect IgG subclass molecules in the NhP. All IgG subclasses also showed a significant response post-primary and secondary immunizations. Although major participation in the IgG response was restricted to IgG1 and IgG3 subclasses, minor contributions of IgG2 and IgG4 subclasses were detected, especially later in the response. Methods of determining IgG avidity were developed and used for NhP serum. The IgG anti-tetanus toxoid avidity was increased significantly by primary and secondary immunization. Finally, the peak IgG antibody avidity was not coincident with peak antibody level activity, although increases in IgG avidity and IgG levels were correlated post-immunization independent of time.

The immunologic response to the protein antigen tetanus toxoid was characterized in the NhP. The results afforded a preliminary look at antibody-protein toxin interactions in a species closely related to man, the nonhuman primate. However, tetanus toxoid is just one of many bacterial protein toxins. Bacterial protein toxins are reputed to be involved in a host of other pathologic processes involving man and animals.

One pathologic entity in which protein toxins may have a role is periodontal disease. Dental microbial plaque has been assigned the primary role in periodontitis etiology as a result of experimental gingivitis studies by Loe (1965) and Theilade (1966). Loesche & Syed in 1976 have suggested that plaque mass was not as important as were the specific bacterial pathogens within the plaque. It was noted that with plaque maturation, certain more harmful species within the plaque gained prominence. These species produced substances which were not only toxic to host cells, but also induced host immune

responses which could cause further destruction. Numerous bacterial associated substances have since been identified with toxic and/or immunogenic implications (Slots 1987) to support this hypothesis.

Localized juvenile periodontitis, a rapidly destructive disease of adolescents, has been associated with a high prevalence of a particular bacterium, *Actinobacillus* actinomycetemcomitans (Slots 1984) in dental plaque, and serum antibody to that microorganism (Ebersole 1982). Further, the most pathogenic strains of *A. actinomycetemcomitans*, and possibly the only strains associated with rapid periodontal destruction of LJP, are those that produce a leukotoxin (Lai & Listgarten 1980, Ebersole 1980b, Listgarten 1981). The leukotoxin (LT) is a heat-labile, protease sensitive, protein toxin with a molecular weight of 115 kDa which is capable of killing human and some monkey PMN's and monocytes, but is not cytocidal for leukocytes in any other animal species (Tsai 1984). It is contained in the periplasmic space of the microorganism (DiRienzo 1985), and is released in vesicles from the microbial surface (Lai 1981). The toxic activity is neutralized by immune serum from LJP patients and also by serum from several different species of animals previously immunized with *A. actinomycetemcomitans* extract. The toxic activity appears to be irreversible for human leukocytes once binding of LT to the cell membrane occurs after a 5 minute exposure of the leukocyte culture to LT (Taichman 1980). The same irreversible reaction with monkey (*Macaca fascicularis*) PMN's and monocytes has also been reported by Taichman et al. (1984).

Other protein toxins such as α-hemolysins of *E. coli* and *P. hemolytica*, and leukotoxin of *A. pleuropneumoniae* are putative factors in pathology of man and other animals. The leukotoxin gene (lktA) from *A. actinomycetemcomitans* was recently cloned and sequenced (Kolodrubetz 1989). The protein encoded by lktA shared at least a 42% identity with a *Pasteurella hemolytica* leukotoxin and α-hemolysins of *Escherichia coli*, and *Actinobacillus pleuropneumoniae*. Another gene, lktC, was linked to lktA of *A. actinomycetemcomitans* and is also thought to be related to the lktC proteins from the other bacteria and shared at least 49% amino acid identity. Although very significant homology exists with the other leukotoxin/hemolysins, the lktA from *A. actinomycetem-comitans* has several unique characteristics including a very basic pI of 9.7, versus 6.2 for

the lktA proteins of the other bacteria. Using cloned genes as probes, Ebersole et al. (in press) reports evidence that a TOX- strain of *A. actinomycetemcomitans* contains the leukotoxin gene but apparently fails to transcribe it at high levels.

A. actinomycetemcomitans has been directly associated with some of the most rapidly progressing forms of periodontitis. Although this organism produces many toxic and immunogenic substances, it appears that only those strains of *A. actinomycetemcomitans* which produce leukotoxin are associated with severe periodontal destruction. Furthermore, those patients in whom the disease "burns out" are found to have increased anti-LT antibody titers. *In vitro* neutralization of the toxin by antiserum has been reported, however not all patients who develop high titers demonstrate arrested disease. Puzzling questions remain to be answered. Why does not all anti-LT serum have adequate neutralizing capabilities? Is control of the disease in healthy patients related to the humoral arm of immunity? If so, can the response be manipulated in favor of the host? Reported neutralizing capabilities of immune serum in periodontal as well as other diseases suggests that control of the damage caused by protein toxins might some day be feasible through immunologic methods. A better understanding of the immune reaction to insult by such protein toxins may eventually allow for immunologic intercession to prevent destruction mediated by these agents. This preliminary study provides a model with apparent immunologic similarities to man, and baseline data towards a better understanding of the relationship of the humoral response to protein toxins in the NhP and, possibly, in man.

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